

STUDIES ON NITRATE REMOVAL IN CONTAMINATED WATER THROUGH AN INTEGRATED LABORATORY APPROACH USING AEROBIC BACTERIAL STRAIN

Submitted in partial fulfilment of the requirements for the award of

Master of Technology degree in Biotechnology

By

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**DEPARTMENT OF BIOTECHNOLOGY
SCHOOL OF BIO & CHEMICAL ENGINEERING**

SATHYABAMA

**INSTITUTE OF SCIENCE AND TECHNOLOGY
(DEEMED TO BE UNIVERSITY)**

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BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of **GOKUL AKASH S (42810002)**, who carried out the project entitled "**STUDIES ON NITRATE REMOVAL IN CONTAMINATED WATER THROUGH AN INTEGRATED LABORATORY APPROACH USING AEROBIC BACTERIAL STRAIN**" under our supervision from November 2023 to April 2024.

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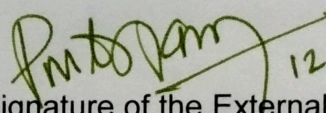
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CERTIFICATE

This is to certify that the dissertation entitled “**STUDIES ON NITRATE REMOVAL IN CONTAMINATED WATER THROUGH AN INTEGRATED LABORATORY APPROACH USING AEROBIC BACTERIAL STRAIN**” submitted to the Sathyabama Institution of Science and Technology, Chennai in partial fulfillment of the requirement for the degree of **MASTER OF TECHNOLOGY IN BIOTECHNOLOGY** is a record of bonafide research work carried out by **Mr. S. GOKUL AKASH (Reg. No: 42810002)** during the period November 2023 to April 2024 of his study in the Department of Microbiology at Periyar University, Salem under my supervision and the dissertation has not formed the basis for the award of any degree/ diploma/ fellowship/ associateship or other similar title to any candidate of any University.

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ABSTRACT

Freshwater is a scarce resource that continues to be at high risk of pollution from anthropogenic activities, requiring remediation in such cases for its continuous use. The agricultural and industrial activities extensively use water and nitrogen dependent products, mainly in fertilizers and explosives, respectively, with their excess accumulating in different water bodies. The intensive use of nitrogen based products and their inappropriate disposal has contributed to ammonia and nitrate contamination of both surface and groundwater source. Ground water contaminated by nitrate will cause direct or indirect harm to human health. Although, removal of nitrate from water and soil through the application of chemical, physical and biological methods has been studied globally, these methods seldom yield N_2 gas as a desired by product for nitrogen cycling. These methods predominantly cause secondary contamination with deposits of chemical waste such as slurry brine, nitrite (NO_2), ammonia (NH_3), and nitrous oxide (N_2O), which are also harmful and fastidious to remove. Biological denitrification is currently the most economical, effective and widely used ground water nitrate removal technology. In this process, nitrate being reduced by a aerobic bacterium to nitrogen oxides or nitrogen (N_2) under aerobic conditions. In this study, nitrate degrading bacterial strains were obtained from bioremediation laboratory of Periyar University. Improvement of microbial strain was done by using mineral salt medium and developed to enhance the degradation of nitrate to nitrite and ammonium. Effects of various parameters such as carbon sources, pH and temperature on denitrification process were also studied. Bioreactor and column approach followed by inorganic coagulation process in the study exhibits significant removal of nitrate from contaminated water. Hence, biological method using a nitrate degrading bacterium could be useful to treat the water contaminated with nitrate.

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1. INTRODUCTION

Nitrate (NO_3^-) concentration in ground water has increased globally. The concentration of nitrate in ground water can be enhanced by shallow ground water table, manures, irrigation and abundant rainfall. Livestock feeding, barnyards, septic tanks, industrial effluents, animal and human contaminations are the other important sources contributing high amounts of nitrate to ground water (Stevenson, 1986; Hem, 1992; Curt *et al.*, 2004). In and around areas of high urbanization and industrialization, municipal and industrial wastes may contribute high levels of nitrate to ground water (Handa, 1983). Ground water with high concentrations of nitrate when used for drinking cause potential risk to public health, particularly to infants (Fewtrell, 2004).

Nitrate concentration in surface water is usually below 5 mg/l of $\text{NO}_3\text{-N}$, however, the World Health Organization found that 31.5% of 2,000 wells surveyed in Canada had concentrations exceeding 20 mg/l $\text{NO}_3\text{-N}$. Health and Welfare Canada has set a maximum acceptable concentration of 10 mg/l $\text{NO}_3\text{-N}$ or 45 mg/l NO_3 (Reddy and Lin, 2000), which is consistent with the USEPA standards. Only about 2.66% of the earth's water resources are fresh water and only 0.6% is available for drinking (Shrimali and Singh, 2001). Therefore, it is essential that water resources be treated as precious.

Excessive use of nitrogenous fertilizers of agriculture has been one of the primary sources of high nitrate in ground water (De Rao, 1980; Schepers *et al.*, 1984). Apart from nitrate, nitrogen is applied in the form of ammonium (NH_4^+) and amide (NH_2^-), which generates nitrate in soil system through mineralization, fairly rapid in tropical and subtropical soils (Majumdar and Gupta, 2000). Due to its high solubility in water and low retention by soil particles, nitrate is prone to leaching to the subsoil layers and ultimately to the ground water, if not taken up by plants or denitrified to N_2O and N_2 . The rate of leaching is governed by the soil properties and amount of water present in the soil system (NIOSH, 1996; Tennehill *et al.*, 1996).

Foster and Crease (1974) first brought the threat due to nitrate in ground water to the public of East Yorkshire. Ground water midlands, East Anglia, Yorkshire and North Kent were also most vulnerable to nitrate pollution. The studies of Foster and Young (1981) demonstrated that considerable variation in nitrate concentration could occur both specially and with depth in aquifers subject to diffuse pollution. High nitrate (34.4 to

155 mg/l) in ground water was a common occurrence in some parts of Southern Africa (Connelly and Taussig, 1994). In Sri Lanka, triple cropping with heavy application of nitrogenous fertilizer resulted in high nitrate contamination (45 to 90 mg/l) of ground water (Chilton *et al.*, 1994). The study of Power and Saikh (1995) indicated that higher concentration of nitrate (0.02 to 95 mg/l) in the sand aquifers of Canada was arisen primarily from fertilizers. In sandy aquifers of USA, the nitrate concentration was also as high as about 64.9 mg/l and the source was mainly from sewage (Hantzscs and Finnemore, 1992).

Gupta (1981) and Gopal *et al.* (1983) have reported high levels of nitrate in ground water notably in Barmer, Bikaner, Jaipur, Jodhpur, Jaisalmer and Udaipur. About 40 % and 74 % of ground water samples in Jaipur and Barmer respectively, were found to have more than 50 mg/l of nitrate. Tanwar (1981) found nitrate concentration ranging from 5 to 60 mg/l in different regions of Haryana. High concentration of nitrate was found in Hissar (1800 mg/l) and Mahendragarh (1620 mg/l) district of Haryana (Handa, 1986). Mishra and Richaria (1999) have reported high nitrate concentration (45 to 60 mg/l) around the flanks of Bichiya and Beehar streams. A high level of nitrate, surpassing >300 with a maximum of 1000 mg/l was observed at several places of Uttar Pradesh (Somasundaram *et al.*, 1993).

Rajasthan is considered as a major nitrate affected state in the country (Gopal and Bhargava, 1982). Nitrate level in ground waters of Rajasthan have caused a great concern particularly in arid and semiarid climates. Nearly the whole Rajasthan is suffering from the problem of high nitrates with concentration ranging from 40 to 1000 mg/l (Sudhir Kumar *et al.*, 2002). Mathur and Ranganathan (1985), found nitrate concentration ranging from 45 to 613 mg/l at Jodhpur city. There are numerous sources in environment that contribute to the total nitrate content of natural waters e.g. atmosphere, geological features, anthropogenic sources, atmospheric nitrogen fixation and soil nitrogen. However, a detailed hydrological investigation conducted in the state has indicated a heterogeneous pattern of nitrate distribution. In sandy soil of Rajasthan with low water holding capacity and high permeability, movement of pollutants like chloride and nitrate is much quicker than in clayey soil. This is probably the main cause for high nitrates in Rajasthan (Sudhir Kumar *et al.*, 2002). They have also reported high

nitrate contamination in some districts of Rajasthan. The nitrate contamination in ground water reported by TWAD Board, Chennai (2001) seems to be very high in some parts of Thiruvannamalai, Salem, Dharmapuri and Erode districts of Tamil Nadu and the concentration were found to be >100 mg/l (Table 1.1). Different organizations and countries have set standards for nitrate in potable water (Table 1.2), to safeguard public health from the hazards associated with high concentration of nitrate. Nitrate causes various problems to human beings, animal, surface water and aquatic life when the nitrate exceeds above the permissible limits.

Large amounts of nitrate in drinking water are a cause of a disease called methemoglobinemia (blue baby disease), a blood disorder primarily affecting infants under six months of age (Plate 1.1). Methemoglobinemia is a condition in which the oxygen carrying ability of red blood cells is reduced. The acutely poisoned person will have a blue discoloration of the skin due to the reduction of oxygen in the blood system and must be attended by a physician immediately (Comly, 1987; Darbi *et al.*, 2002). The development of methemoglobinemia from nitrate in drinking water is dependent upon the bacterial conversion of nitrate to nitrite.

Investigations have confirmed that the infants gastric juices being at relatively higher pH, facilitate the bacterial conversion of nitrate to nitrite (Comly, 1987; Fonseca *et al.*, 2000). Nitrite is consequently absorbed by haemoglobin to form methemoglobin. High nitrate reduces assimilation of iodine by human body causing goiter but is yet to be proved (ECETOC, 1988). Dorsche *et al.* (1984) and Arbuckle *et al.* (1988) had suggested that high nitrate intake with drinking may lead to the birth of a malformed child. There are reports of other health disorders namely hypertension (Malberg *et al.*, 1978), increased infant mortality (Super *et al.*, 1981), lymphatic cancer (Jensen, 1982), non - Hodgkins lymphoma (Weisenberger, 1993), gastric cancer (Lee *et al.*, 1995), goiter (Sefner, 1995), stomach cancer (Forman *et al.*, 1985; Payne, 1993; Cantor, 1997), thyroid disorder (Schubert *et al.*, 1997), cytogenetic effects (Prakash Rao and Puttana, 2000) and birth defects (Dorsche *et al.*, 1984; Kar *et al.*, 2002). Some studies in Rajasthan revealed recurrent respiratory tract infection and diarrhoea in human beings, besides recurrent stomatitis (ICMR, 1996). Sometime nitrate may also affect health of livestock (Crowley *et al.*, 1974) and aquatic fauna (Kincheloe *et al.*, 1979;

Hecnar, 1995). Hence, removal of nitrate is essential from nitrate contaminated water before being utilized.

In the absence of effective measures to remove nitrate pollution, it was felt that it would be more appropriate to use aquatic plants, aerobic denitrifying organisms and suitable chemical coagulants to tackle the problems of nitrate pollution. Hence this present study was made with the following objectives:

1. To assess and identify the presence of nitrate reducing bacteria obtained from laboratory stock culture.
2. To screen nitrate reducing bacteria by nitrate reduction test using potassium nitrate broth.
3. To find out the effect of carbon sources and temperature on the reduction of nitrate from synthetic water and
4. To study the nitrate removal in synthetic water through a bioreactor and column approach
5. To estimate the efficiency of nitrate removal from synthetic water using chemical coagulants.

Table 1.1. Nitrate contaminated areas in Tamil Nadu Districts

Nitrate affected areas	NO ₃ level (mg/l)
1. Thiruvannamalai District	
a) Vandavasi	> 100
2. Vellore District	
a) Katpadi	45 - 100
b) Tirupatur	> 100
3. Dharmapuri District	
a) Dharmapuri	> 100
b) Krishnagiri	45 - 100
c) Bargur	45 - 100
d) Jerkanikottai	> 100
4. Salem District	> 100
5. Erode District	
a) Erode	> 100
b) Sennimalai	> 100
6. Karur District	
a) Karur	45 - 100
7. Thiruvarur District	
a) Thiruvarur	> 100

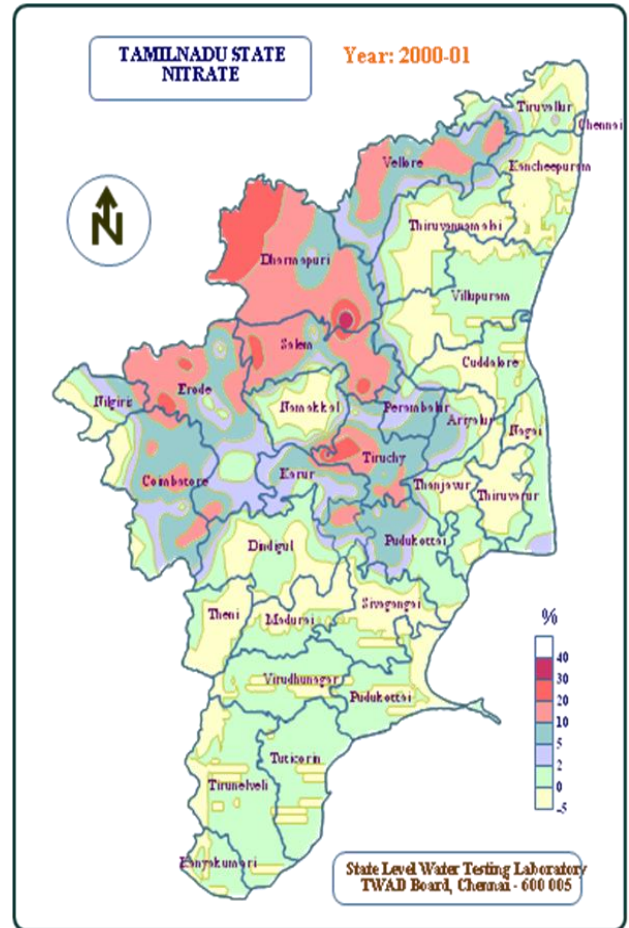


Table 1.2. Maximum permissible limits of nitrate in potable water prescribed by different countries and organization

Country / Organization	Concentration as NO₃ (mg/l)	Concentration as NO₃ N (mg/l)	Remarks
WHO	45	10	Lunkad, 1994
US - EPA	45	10	Lunkad, 1994
ICMR (India)	45	10	Lunkad, 1994
Canada	45	10	Lunkad, 1994
Poland	45	10	Lunkad, 1994
EEC	50	11.3	Lunkad, 1994
Bulgaria	30	6.7	Lunkad, 1994
Belgium	50	11.3	EEC, 1977
Denmark	50	11.3	WHO, 1985
Finland	30	6.8	WHO, 1985
Hungary	40	9.0	WHO, 1985
UK	50	11.3	EEC, 1977
USA	45	10	EEC, 1977

Plate 1.1. Diseases and environmental problems due to nitrate contamination

(a) Methemoglobinemia



(b) Goiter in human being



(c) Goiter in animal



(d) Aquatic fish kill



(e) Eutrophication



2. REVIEW OF LITERATURE

2.1. About nitrate contamination

The wide range of pollutant sources and the complexity of recharge in urban areas make the estimation of pollutant loads difficult task in these areas. This is also true for oxidized and reduced forms of nitrogen that are present in many possible recharge sources of urban aquifers. They include sewage, septic tanks, industrial spillages, contaminated land, landfills, river or channel infiltration, fertilizers used in gardens, house building, storm water and direct recharge. Because of the number of nitrogen sources in urban areas, it is not surprising to find elevated nitrogen concentrations in urban aquifers. The sources of nitrogen in the urban environment are a mixture of point sources (e.g. landfills), multipoint sources (e.g. soakways and leaky sewers) and diffuse sources.

There are many sources of nitrogen that could potentially lead to the pollution of the groundwater with nitrates. The anthropogenic sources are really those that most often cause the amount of nitrate to rise to a dangerous level. Waste materials are one of the anthropogenic sources for nitrate contamination of groundwater. Many local sources of potential nitrate contamination of groundwater exist such as sites used for disposal of human and animal sewage, industrial wastes related to food processing, munitions, some polyresin facilities, and accidental spillage of nitrogenous materials (Hallberg and Keeney, 1993). Landfill leakage is the quickest growing supply of nitrate to groundwater in China. Rapid urbanization and industrialization are the primary driving forces for the growth of landfill leakage (Mor *et al.*, 2006).

2.1.1. Non-Agricultural sources of nitrate

One potentially large source of nitrogen pollution of groundwater is the application of nitrogen-rich fertilizers to turfgrass. This occurs on golf courses and in residential areas. There are five fates for this nitrogen once it is applied to turfgrass. It may be: i) taken up by plants ii) stored in soil iii) lost to atmosphere iv) lost to groundwater and v) lost to runoff. Many studies have shown that most of the nitrogen, about 30 to 50 percent, is taken up by the plant. According to the study by United States Golf Association only one to two percent of the nitrogen is leached beyond the root zone (Bocher and ward, 1995). Nitrate has been connected to agricultural sports due to the

usage of fertilizers. However, there are other nitrate Resources related to urban development that may boom Nitrate concentrations in groundwater. Studies in the Previous few years have located that nitrate concentrations in A few city aquifers are comparable or even higher to those of their surrounding agricultural areas (Lerner *et al.*, 1999).

2.1.2. Agricultural sources of nitrate

Agriculture is the largest nitrate source, accounting for about 90% of that which leaches to groundwater. The remaining 10% comes from septic tanks, sludge disposal and other sources (Shaw and Byron, 1994). Farmers first deplete the soil by excessive and repetitive planting and then try to replenish the resulting less-productive soil by putting more and more nitrogen-based fertilizer on the land to keep crop yields constant.

The problem caused by farms results from the grazed grasslands and feedlots. In grazing pastures, animal wastes are concentrated in small pastures, and this leads to inefficient use of nitrogen and causes the potential for groundwater contamination by nitrate. This problem is even worse in Europe where grazing pastures are usually more intensively fertilized than in the U.S., therefore more nitrate can be leached to the groundwater (Hallberg and Keeney, 1993). Even small farms can contribute to the problem of excess nitrates because of high concentrations of manure that they may have in the barnyard or feedlot areas (Hallberg and Keeney, 1993).

The more usage of nitrogen fertilizer, the greater the chance of nitrate contamination of groundwater. Nitrogen is applied in the form of ammonium and amide, which generate nitrate in soil system through mineralization. It is fairly rapid in tropical and subtropical soils (Majumdar and Gupta, 2000). Due to its high solubility in water and low retention by soil particles, nitrate is prone to leaching to the subsoil layers and ultimately to groundwater, if neither taken up by plants nor denitrified to N_2O and N_2 . Farmers still consider nitrogen fertilizer as cheap insurance against crop failure. Approximately one dollar worth of fertilizer could bring in ten dollars of corn if the soil has a lack of nitrogen. So, farmers would rather add too much nitrogen than too little. It is very difficult to determine exactly how much nitrogen a crop will need before harvest time due to yearly change in crop yields and weather conditions. Even if farmers cut down on nitrogen fertilizer, there will still be some nitrate leaching.

The primary N input to United Kingdom groundwater is derived from manures, Fertilizers sewage sludges and crop residues in agricultural regions (DEFRA, 2006). Nitrogen fertilizers are the essential source of water-soluble nitrate and Nitrite compounds inside the soil that may be over excited through floor runoff into groundwater, rivers and ingesting water (Shukla and Saxena, 2020). Discount of N fertilizer efficiency is realized as first hassle through formers. In regards to the world average fertilizer application of 132kg N ha¹ in 2005/2007 (Alexandratos and Bruinsma, 2012). The leached nitrate reaching to floor and shallow groundwater tables has made the drinking water infected through nitrates exceeding the same old beneath agricultural fields in specific components of the arena (Keeney and Follett, 1991) & (Carneiro *et al.*, 2012). Nitrate in nature is normally fashioned as a denitrification product of nitrogen. It's miles surprisingly soluble in water and is unreactive. Fertilizers and other waste products that include nitrogen are decomposed to provide Ammonia observed by way of similarly oxidation to nitrate and nitrite. Nitrate is usually present in air, water, and food and is taken up through plants to synthesize natural nitrogenous compounds (2011) Geneva.

Nitrate is one of the most significant pollutions of groundwater in lots of developed and developing nations. Extensive use of chemical fertilizers, manure in crop production, and poor wastewater Treatment and series structures are the main assets of nitrate infection in groundwater (Mishra, 2009). Nitrate contamination related to anthropogenic activities are particularly derived from both diffuse (non-point) resources, which includes livestock and artificial and organic fertilizer software, and point sources, including septic systems and/or waste water treatment plant (WWTP) effluents (Peed, L.A; 2011). Global food safety owes a good deal to the considerable use of mineral fertilizers.

However, the commercially to be had fertilizers are blended with several hint metals, which can be delivered into the soil together with the utility of fertilizers (Westfall *et al.*, 2005). Among inorganic fertilizers, phosphate fertilizers are the fundamental supply of contaminants as they may comprise strains of Cd, Pb, As, Cr, fluorine (F), strontium (Sr), thorium (Th), uranium (U), zinc (Zn), and so forth (Raven *et al.*, 1998).

2.2. Natural sources of nitrate

Natural sources also contribute high concentration of nitrates to groundwater. One example is the effect of forested areas on the leaching of nitrates to the groundwater. Mature forests conserve nitrogen but human disturbances can lead to nitrate pollution of groundwater. However, forests represent a very small source of nitrogen compared to agriculture (Hallberg and Keeney, 1993). Crop yields are laid low with many elements related to climate Alternate consisting of temperature, rainfall, atmospheric CO₂ concentration, excessive climate activities and climate variability (RR, 2009).

2.2.1. Industrial sources

Many industries generate high nitrate-bearing wastewaters. Industries producing basic organic chemicals, fermentation products, fertilizers like calcium, potassium, sodium, nitrates, nuclear power, stainless steel, nitroglycerine, trinitrotoluene etc., generate high nitrate-containing wastewaters. Indirect sources are wastewaters emanating from dairy, slaughter house, food and meat processing etc., giving rise to concentrated organic nitrogenous wastewaters. In the final stage of aerobic stabilization of such wastewaters, organic or inorganic nitrogen is ultimately converted into nitrate as end product of metabolism (Deshmukh, 1992).

Water pollution is a first-rate difficulty with numerous consequences on existence on this planet. It's far specifically because of the industrial pollutants and anthropogenic sports (Khatri, 2016). Two industrial wastewaters originating from an ice-cream production manufacturing facility and a beet-sugar manufacturing unit, Obtaining a mean unique denitrification rate of 3.28 mg NO₃-N/g VSS h and a couple of 72 mg NO₃-N/g VSS h, respectively (Cappa; 2004).

2.3. Effects of nitrate

There are reports of some human health disorders namely methemoglobinemia, non- Hodgkins's lymphoma, birth defects, hypertension, goiter, anemia, stomach cancer, lymphatic cancer and gastric cancer and some time it may also affect livestock health, aquatic life and surface water. Nitrate additionally alters the functioning of the thyroid gland that may intervene with ordinary boom, improvement, and survival of freshwater organisms (Guillette and Edwards, 2005).

2.3.1. Methemoglobinemia (Blue-baby Syndrome)

Nitrate can cause significant health problem to human beings. High concentration of nitrate in drinking water often causes disease called methemoglobinemia (Blue baby syndrome), a blood disorder primarily affecting infants under six months of age. Methemoglobinemia is the most common among infants and is potentially the most serious complication of nontherapeutic, excessive nitrate and nitrite (Frank *et al.*, 2005).

Methemoglobinemia is the condition in the blood which causes infant cyanosis or blue-baby syndrome. Human babies are extremely susceptible to acute nitrate poisoning because of certain bacteria that may live in their digestive system during the first few months of life. These bacteria change nitrate into toxic nitrite (NO_2). The nitrite reacts with hemoglobin (which carries oxygen to all parts of the body) to form methemoglobin which does not carry oxygen. One nitrite molecule then reacts with two molecules of hemoglobin to form methemoglobin. As the oxygen level decreases, the body is suffocated. This condition is called methemoglobinemia. In acidic condition, the reaction occurs quite rapidly (Comly, 1987). This altered form of blood protein prevents the blood cells from absorbing oxygen which leads to slow suffocation of the infant which may lead to death (Kar *et al.*, 2002). Because of the oxygen deprivation, the infant will often take on a blue or purple tinge in the lips and extremities, hence so-called blue baby syndrome (Lukens, 1987). Other signs of infant methemoglobinemia are gastrointestinal disturbances such as vomiting and diarrhea, relative absence of distress when severely cyanotic but irritable when mildly cyanotic, and chocolate-brown colored blood (Johnson *et al.*, 1987).

Since 1945, there have been over 2000 cases of infant methemoglobinemia reported in Europe and North America with 7 to 8 percent of the afflicted infants dying (Rail, 1989). However, problems can be severe as shown in a 1950 report, there were 144 cases of infant methemoglobinemia with 14 deaths in a 30-day period in Minnesota (Johnson *et al.*, 1987). In Hungary there were over 1300 cases severely affected by methemoglobinemia between 1976 and 1982 (Addiscott *et al.*, 1991). This was an isolated case. However, it shows that nitrate concentrations in wells can increase to deadly levels rapidly and therefore the issue of nitrate contamination should not be ignored.

Although many studies have been performed to make an attempt to link stomach and gastrointestinal cancer to nitrate intake, there has been yet no conclusive evidence of a correlation between them. In fact, two particular studies in the United Kingdom showed an inverse relationship where occurrence of stomach cancer was the highest in areas where concentration of nitrate in groundwater was the lowest and vice versa (Payne, 1993). Scientists claim that nitrate can cause a potential risk because of nitrosation reactions which form N-nitroso compounds in the presence of appropriate substrates, which are strongly carcinogenic in animals (Forman *et al.*, 1985). In other areas of the world such as Columbia, Chile, Japan, Denmark, Hungary and Italy, similar studies have suggested a correlation between them, although no concrete evidence to support this theory exists (Forman *et al.*, 1985). At present, no other toxic effects have been observed under conditions of high nitrate levels. Even at exposure to levels of 111 mg/l there were no adverse conditions in infants except for methemoglobinemia (Gustafson, 1993). Other claims that intake of groundwater contaminated with nitrate is linked to birth defects, hypertension and high blood pressure in adults. Health danger assessment is one of the most effective gears to estimate the human health hazard on the idea of intake, dermal touch, and inhalation. But, on this examine oral intake of consuming water turned into concerned as a primary publicity pathway, and fluoride and nitrate concentrations were decided on because the parameters for fitness risk evaluation. Unites States Environmental safety business enterprise considered those nitrate parameters as non-carcinogenic dangers for human fitness (USEPA 1991).

2.3.2. Goiter and birth defects

High nitrate intake can reduce the body assimilation of iodine and thus causing goiter. At present, only a few studies are available. Whether exposure to nitrate is an ethiological factor in human goiter or not remains to be determined. It was also suggested that an increased intake of nitrate from drinking water carries an increased risk for bearing a malformed child (Kar *et al.*, 2002). Animal research display that ingestion of nitrate at high doses can competitively inhibit iodine uptake and set off hypertrophy of the thyroid gland (Groef, 2006). An early examine of girls inside the Netherlands ingesting water with nitrate degrees at or above the MCL, located accelerated incidence of thyroid hypertrophy (Van Maanen; 1996).

2.4. Nitrate removal

The increasing concern with high concentrations of nitrate in drinking water has led to the development of various methods for efficient nitrate removal. Nitrate is a stable and highly soluble ion and has a low potential for adsorption or coprecipitation (Kapoor and Viraraghavan, 1997). These properties are responsible for difficulty in nitrate removal using conventional water treatment processes including lime softening and filtration. There are several technologies developed for nitrate removal from drinking water are reverse osmosis (Tannehill *et al.*, 1996), ion exchange (Kim and Benjamin, 2004), ion exchange membrane bioreactor system (Velizarov *et al.*, 2002 a and b), catalytic reduction (Reddy and Lin, 2000), electrolysis (Elmidaoui *et al.*, 2001), activated carbon process (Sison *et al.*, 1996), land disposal (Deshmukh, 1992), distillation (Uchida *et al.*, 1996), chemical denitrification (Hu *et al.*, 2001), wetland remediation (Ingersoll and Baker, 1998), phytoremediation (Sanderson *et al.*, 2001), microbial treatment and especially for anaerobic treatment (Natcheva and Beschkov, 2003).

Nitrate discount may additionally occur via plant uptake, mineralization-immobilization techniques, volatilization, losses with the aid of run-off and denitrification (Shindo & Nishio 2005; Chen *et al.* 2014). The ex-situ remedy option often entails the P&T approach along with extraction of infected groundwater, remedy and discharging the water lower back into the aquifer through reinjection or percolation. This approach has been used in superfund web sites within the u.S. For remediation of organic polluted aquifers. This application is only when contaminant plume may be nicely defined (U.S. EPA 2005; Faris 2009; King *et al.* 2012). Stable, liquid and gaseous nation electron donors will also be used to achieve heterotrophic Denitrification. Liquid nation electron donors consist of: acetate, format, sucrose, methanol, ethanol, Starch, molasses (Ayyasamy *et al.* 2007). The most generally used treatment methods to remove/lessen NO₃ – include chemical denitrification the usage of zero-valent iron (Fe⁰) (Ahn, 2008).

2.4.1. Physical Methods

2.4.1.1. Reverse Osmosis

In a reverse osmosis process, ionic species present in water are removed by forcing the water across a semi permeable membrane and leaving nitrates and other

ionic species behind. Removal of nitrates is achieved by subjecting water in reverse osmosis cells to pressures exceeding its corresponding osmotic pressure. Pressures ranging from 300 to 1,500 psi (2,070 kPa -10,350 kPa) are applied to reverse the normal osmotic flow of water. The membranes used in this procedure are commonly composed of cellulose acetate whereas the membranes made of polyamides or composite membranes are used to a lesser extent. These membranes do not have a preference for any particular ion, but the amount of salt rejected is proportional to the valence of the ions in solution (Kapoor and Viraraghavan, 1997). There are problems resulting from deposition of soluble materials, organic matter, suspended particles, pH variations and chlorine exposure. Therefore, pretreatment is a necessary procedure which will often extend the life of the membrane and improve efficiency. Typical pretreatment includes adding sodium hexametaphosphate for carbonate and sulfate control as well as filtration and lowering of pH by addition of acid. Post treatment steps are also required, which include air stripping for stabilization, pH adjustment to control corrosion and chemical addition for disinfection. The semi-permeable membrane can be expected to remove nitrates efficiently for three to five years (Tannehill *et al.*, 1996). Schoeman and Steyn (2003) applied reverse osmosis technology for water denitrification in a rural area. The results revealed nitrate nitrogen was reduced from 42 mg/l to less than 1 mg/l.

The major disadvantage of this method is the disposal of the super concentrated ion solution that is produced. The disposal options depend on conditions of federal, state, and local regulations. The options include discharge to lakes and streams or oceans, deep well injection, lined evaporation lagoons, irrigation, and dilution in wastewater systems (Tannehill *et al.*, 1996). About 65% of nitrate separation was observed for influent NO_3^- - N concentrations of 18 - 25 mg/l (Guter, 1982). It was also found that 35% of the total influent water became waste brine solution. Compared to ion exchange, reverse osmosis is estimated to be three times more expensive.

Much like ion trade technology, opposite osmosis may be used for saline nitrate impacted groundwater. For example, opposite osmosis era has been broadly applied for nitrate Removal from consuming water in South Africa (Schoeman & Steyn 2003). Reverse osmosis (RO) era is used in pump and treat (P&T) packages to take away

nitrate from groundwater. Within the RO procedure, infected groundwater is extracted and pumped via opposite osmosis cells under excessive strain (ca 300–1,500 psi) (Archana *et al.* 2012). Reverse osmosis (RO) become taken into consideration as ex- and in situ packages for the reduction of nitrate from Drinking water. Opposite osmosis can be used to lessen a huge variety of contaminants simultaneously (Black. 2013).

2.4.1.2. Ion Exchange

Ion exchange involves exchange of ions in water and exchange resin. An anion exchange resin consists of a polymer of positively charged sites that are bonded to negatively charged ions. In this case sodium chloride is generally used to charge the column. When sodium chloride is flushed through the resin column, chloride ions bind to the positively charged sites on the resin. Then water containing nitrate is passed through the column and the nitrate ions, which are negatively charged, displace the chlorine ions on the resin (Boumedieence and Achour, 2004).

Clifford and Liu (1993) conducted a study to estimate nitrate removal from drinking water by ion exchange. The raw water contained 18 to 25 mg/l $\text{NO}_3\text{-N}$, 43 mg/l sulfate and 530 mg/l TDS. The process reduced the nitrate level to below 10 mg/l. The problems associated with ion exchange include disposal of the spent brine containing nitrate and excess sodium chloride. This causes corrosion and negative health effects due to the increase of chloride content in the treated water (Kapoor and Viraraghavan, 1997).

Leakovic *et al.*, (2000) reported a treatment process in fertilizer industry wastewater by an ion exchange process using strong acid cation and weak basic anion exchangers. The wastewater in the investigation period contained a high content of nitrogen in ammonium form (with an average of 325 mg/l N-NH_4^+) as well as nitrogen in nitrate form (with an average of 201 mg/l N-NO_4^-). After treatment, the wastewater contained 8.7 mg/l of ammonium nitrogen and 7 mg/l of nitrate nitrogen and with the conductivity of $150 \mu\text{S cm}^{-1}$. There are several advantages of ion exchange which includes reuse of treated wastewater, utilization of product regeneration for fertilizer and absence of other pollutants (Leakovic *et al.*, 2000). This method has disadvantages of unsatisfactory anion resin conditions due to the production of nitric acid and unsatisfactory vacuum evaporation conditions (Leakovic *et al.*, 2000). Another problem was that the resin can be often fouled due to the presence of organic matter in the

influent (Kapoor and Viraraghavan, 1997). However, the resin can be restored, however, with the addition of bentonite clay to backwash water. Precipitates of iron and silica can reduce the nitrate removal capacity of the resin.

Ion exchange processes had been utilised in groundwater remediation in many components of the sector. Strong base anion exchange resins offer web sites for the exchange of chloride and bicarbonate ions with nitrate from the aqueous infected water (Nujic *et al.* 2017). The utility of ion exchange technologies the usage of strong base exchange resins of trimethylamine useful groups confirmed a selective ion trade affinity within the order bicarbonate < chloride < nitrate < phosphate. Those resins have been particularly designed with a high range of carbon atoms Around ammonium nitrogen functional companies to boom the nitrate selectivity (Samatya *et al.* 2006). The governing basic in ion trade (IX) approach is generally replacement chloride anions with now not applicable compound inclusive of contaminants, total dissolve solids and so on. The process of nitrate discount with ion exchange is just like water softening, however the distinction among them is polymer resin (Seidel; 2011).

2.4.1.3. Catalytic Reduction

Horold *et al.*, (1993) revealed a process that palladium-alumina catalysts were effective in the reduction of nitrate to nitrogen and ammonia in the presence of hydrogen. In addition, the lead, copper, and aluminium oxide catalyst showed a complete removal of nitrate from water having an initial concentration of 100 mg/l nitrate. The process could be easily automated, operated and may be useful for small water treatment system. This process is still in the development stages, and the associated kinetics and long-term performance of catalysts have not been studied (Kapoor and Viraraghavan, 1997). Reddy and Lin (2000) reported that catalytic process has a potential to remove nitrate from groundwater in small agricultural communities for treatment of drinking water. This technique has a potential for field application because rhodium can be coated onto fiberglass mesh and a photovoltaic cell can supply the redox potential. This method can become quite inexpensive since sunlight can be used as an energy source.

To triumph over the problem of high production of NH_4^+ ions during the direct discount of nitrate by using ZVI and NZVI without H_2 supply, the H_2 precipitated-

catalytic reduction era by means of coating semi conductive metals (i.e., Pd, Cu, Ni) on the surface of ZVI has been studied to decorate N₂ selectivity (Liu; 2014).

2.4.1.4. Electrodialysis

Electrodialysis (ED) is like reverse osmosis in many ways. In electrodialysis, ions are transferred from a solution of lower concentration to a solution of higher concentration due to application of a direct current. Electrodialysis selectively removes undesired ions as water flows through a semi-permeable membrane. Similar to reverse osmosis, the water must be pretreated. In electrodialysis reversal (EDR) process, the polarity of two electrodes is reversed two to four times an hour to change the direction of ion flow. This process reduces scaling and chemical usage compared to conventional ED and has been used to produce drinking water from brackish and seawater (Menkouchi Sahli *et al.*, 2006). Nitrate removal efficiency is similar to that of reverse osmosis, but electrodialysis is limited to treat soft water with less acid dosages and higher water recovery rates. This process is currently considered too expensive (Kapoor and Viraraghavan, 1997). Miquel and Oldani (1991) developed a selective nitrate removal process based on electrodialysis. They achieved effective in reducing nitrate concentrations from 50 to less than 25 mg/l without the addition of any chemicals. In other experiment Menkouchi Sahli *et al.* (2006) conducted an electrodialysis operation for technical optimization of nitrate removal from ground water using a pilot plant having a capacity of 24m³/d and found. They observed nitrate level below the admissible limits from the concentration of 70 mg/l of nitrate.

Electric present day is the governing basic in electro-dialysis approach (ED) to take away or to lessen pollutants from water. The device includes cathodic and anionic semi-permeable membrane and two electrodes. Electric contemporary causes anions and cations in answer to move towards anode and cathode, respectively. At some stage in the test, ions lure in waste flow and leaving de-mineralized water in the back (Seidel, 2011).

2.4.1.5. Activated Carbon Process

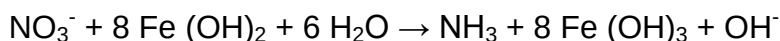
Sison *et al.*, (1996) proposed a denitrification process using granular activated carbon (GAC) column with dynamic modes of external carbon addition. This process is suitable for water or wastewater containing NO₃ but not for organic matters. It was

demonstrated that 87% of nitrate was removed by adding the organic matters once a day with continuous inflow of 20 mg/l nitrate. Ozturk and Bektas (2004) reported a nitrate removal study from aqueous solution using sepiolite, sepiolite activated by HCl, slag and powdered activated carbon were used as adsorbent with a particle size between 71 and 80 μm . Influence of contact time, pH and adsorbent dosage were also investigated using the adsorbents except slag. They found that sepiolite has good nitrate adsorption properties due to its channel structure, high surface area and sorption capacities. The maximum removal for nitrate was observed at pH 2 for activated carbon. pH did not affect nitrate removal significantly for other adsorbents.

2.4.2. Chemical Methods

2.4.2.1. Chemical Denitrification

Reduction of nitrate can also be induced under basic conditions according to the following reaction (Kapoor and Viraraghavan, 1997):



It has been demonstrated that a Fe: NO_3^- ratio of 15:1 is needed in the presence of copper catalyst for the reaction to occur. Use of iron, however, has some disadvantages such as generation of large amount of iron sludge and production of ammonia that requires removal by air stripping. Aluminum has also been used to reduce nitrate. Ammonia was found to be the principal reaction product (60-95%) which should be removed by air stripping. The optimum pH level was found to be 10.25 (Murphy, 1991). Water treatment plants can effectively use lime, which reduces additional costs of raising the pH, for the process of reactions. Experimental results showed that only 1.16 g of aluminum was required for the reduction of 1 g of nitrate, making this process more economical than that with iron. However, this process is still too expensive to implement in a large scale (Kapoor and Viraraghavan, 1997).

Chemical reduction of nitrate (Hecke *et al.*, 1990) and nitrogen oxide (Hu *et al.*, 2001) using ferrous iron as bulk reducing agent has been investigated. Using Fe^{2+} produces voluminous amount of sludge since eight moles of ferric hydroxide is generated per mole of nitrate reduced (Sorg, 1978). In recent years, zero valent iron has been intensively studied for its ability to reduce chlorinated solvents and has been successfully applied to remediate groundwater (Orth and Gillham, 1996). Huang *et al.*,

(1998) developed a denitrification process from aqueous solution using metallic iron at the pH 2 to 5. Rapid nitrate reduction from 50 to 48 mg/l was observed only at pH 2. Acidity is the principal factor which controls the rate and the extend of nitrate removal by Fe^0 . The quick reduction of nitrate at low pH was most likely due to either direct reduction by Fe^0 or indirect reduction by surface hydrogen derived from proton. Diverse chemicals have been used to lessen nitrate form infected water. Among those, the chemical substances like zero-valent iron nano-alumina¹⁷ and magnesium are most Not unusual. Description of different chemical denitrification is given below (Mortazavi, 2010).

2.4.3. Biological Methods

2.4.3.1. Wetland Remediation

Various biological technologies have been established in order to solve the problem of nitrate in drinking water and groundwater. One method to treat a large quantity of groundwater through denitrification is to use wetlands. Wetlands are efficient in removing nitrate from water because of two environmental characteristics that promote denitrification, which are anoxic sediments and a healthy supply of carbon from plant growth (Ingersoll and Baker, 1998). Wetlands have been constructed for several different pollutants from various sources such as sewage effluent, mine drainage and urban runoff (David *et al.*, 1997).

The majority of the organic carbon required for denitrification of wastewater in wetlands is supplied by the wastewater itself. However, in some cases where dissolved organic carbon (DOC) of groundwater is very low, plants are needed to supply the carbon required. Therefore carbon supply would be a limiting factor in wetlands constructed for nitrate removal from water with low DOC (Ingersoll and Baker, 1998). Ingersoll and Baker (1998) showed that the limiting ratio of carbon to nitrogen is approximately 5:1. It was also discovered that nitrate removal efficiency increased as the C: N ratio increased to 5: 1. A key finding was that the reaction rate constant for nitrate was directly dependent upon the addition rate of carbon. These findings suggest that the performance of the nitrate-treatment wetland would be enhanced by promoting plant growth and consequently the carbon supply. This can be achieved by optimizing the habitat for maximum plant productivity such as fertilizing the plants with phosphorus or cutting the plants and leaving the clippings. Constructed wetlands can be used for

remediation of nitrate-polluted aquifers. The wetland would remove the nitrate through denitrification but produce elevated DOC. This method would be the most cost-effective where the groundwater is shallow and the wetlands are situated in a temperate climate. Robins *et al.*, (2000) developed a denitrification study from synthetic ground water using a constructed wetland model. They found adverse nitrate removal from the synthetic feed concentration of 30 mg/l with starch and cellulose mixture as the carbon source.

Wetlands are amongst the ecosystems maximum seriously threatened by way of human interest, which is leading to lack of biodiversity and impaired atmosphere characteristic (Revenga *et al.*, 2005). Currently, expanded loading of shallow lakes with nitrate has been related to loss of macrophyte variety (James *et al.*, 2005). Nitrate removal from groundwater through organic processes has been broadly studied and applied to put off nitrate from infected water. Autotrophic bacteria utilise inorganic carbon sources and electron donors which include bicarbonates, carbon dioxide and sulphur compounds (S^0 , H_2S , S_2^{2-} , $\text{S}_2\text{O}_3^{2-}$, $\text{S}_4\text{O}_6^{2-}$ and SO_3^{2-}) for cellular synthesis (Qambrani *et al.*, 2013). In a natural heterotrophic reactive media, dissolved organic carbon, soluble and bioavailable carbon in pore waters of limited aquifer media can be used for denitrification. However, organic carbon insufficiency may limit the denitrification charge in such environments (Hiscock *et al.*, 1991). Rivett *et al.*, (2008). Dissolved natural compounds evidently present in groundwater are only capable of help small denitrification rates and accordingly, end up the proscribing sellers in the denitrification procedure (Rivett *et al.* 2008).

2.4.3.2. Ion Exchange Membrane Bioreactor

This technique combines ion-selective membrane dialysis (ion exchange) with biological remediation. A nonporous membrane is an essential part of a bioreactor, which keeps water being treated separated from both the microbial culture and the bio medium where the culture is suspended (Fonseca *et al.*, 2000). The possibility of bacterial transfer between treated water and bioreactor depends on the characteristics of the membrane. The membrane can be selected to facilitate the removal of ionic pollutants from the water and to retard the transfer of other inorganic and organic contaminants in the bio medium. A combined form of an anion-exchange membrane and an anoxic denitrifying bioreactor is used. The removal of nitrate is accompanied by

the counter transport across the membrane of a second ion, equivalent in molality, known as the counter ion (Velizarov *et al.*, 2002).

The advantages of this combined technique include prevention of secondary pollution of the treated water with particulate, dissolved organic and inorganic substances. Microbial contamination is also prevented due to the bacteria segregation in the bioreactor. High nitrate removal (approximately 98% with a current of 20mA) was achieved due to facilitated transport through the membrane with less brine solution to be disposed (Islam and Suidan, 1998). Velizarov *et al.*, (2002b) obtained 87% nitrate removal efficiency from polluted water containing nitrate at a concentration of 150 mg/l using ion exchange membrane bioreactor. Recently, the authors Ergas and Rheinheimer (2004) applied similar technique in drinking water containing 200 mg/l of nitrate and found 99% removal efficiency.

The major disadvantage of this process is higher hydraulic residence time (HRT = 4.4h) of the water stream compared with other biological treatment processes. In addition, it was observed that a biofilm developed on the biomedium side of the membrane hindered the transport of nitrate from the water to the biological compartment. The impact of biofilm on nitrate degradation rate and the transmission of ethanol to the water stream should be clarified before this technique can be applied to a large-scale process (Fonseca *et al.*, 2000).

Denitrification reactions can be done using membrane bioreactors. Traditional bio-denitrification techniques were mentioned as a value effective, selective and feasible alternative for dealing with nitrate infected groundwater (Gomez *et al.* 2002). Membrane-bioreactors are used to mitigate those conditions by using allowing nitrate infected Groundwater to skip via the lumen of a microporous membrane. Denitrification occurs at the shell aspect of the membrane as nitrate ions diffuse through the micropore (Fonseca, 1999).

This configuration is same to the extractive procedure besides microporous membrane Era is changed with the aid of dense ion-change membrane technology, the gain of which is That the nonporous membrane enables extra particular extraction of nitrate from uncooked water and in precept hinders the transfer of organic and inorganic pollution present in the bio medium (Fonseca, 2000). Ethanol as the electron donor and

eventually opted for a membrane with the bottom permeability to ethanol. The subsequent obvious diffusion coefficient for ethanol thru the membrane was discovered to be nearly 3 orders of magnitude decrease than that of ethanol in water (Fonseca, 2000).

2.4.3.3. Microbial Denitrification

A great deal of effort has been expended by sanitary engineers to establish empirical methods of tertiary treatment, which will lead to the elimination of much or most of the nitrogenous compounds present in influent/ secondary wastewater. Method of tertiary treatment has been developed over many years along several lines of solution. However, literature on biological nitrification and denitrification for removal of nitrogenous compound is very scarce at present. A review of the published literature on treatment of nitrogenous waste in general biological nitrification and denitrification in particular is made.

Ye and Thomas (2001) reported the pathway of microbial denitrification process (Fig. 2.1). Nitrate is converted to nitrite by assimilatory or dissimilatory nitrate reductases. Assimilatory reduction of nitrate to ammonia via nitrite enables microbes to use nitrate as the nitrogen source. Under oxygen-limiting conditions, nitrite can be reduced to nitric oxide or ammonia. Bacteria with the complete denitrification pathway catalyze the dissimilatory reduction of nitrate to nitrogen.

In some bacteria, dissimilatory reduction of nitrate to ammonia via nitrite can support anaerobic growth or dissipate excess reducing power. Ammonia oxidizers oxidize ammonia to hydroxylamine, which is subsequently converted to nitrite. This process also leads to the production of nitric oxide and nitrous oxide. This pathway clearly indicates that the microbes are the major role to metabolize nitrate either aerobic or anaerobic condition. Based on this metabolic pathway, several authors have been achieved nitrate removal from and wastewater using microorganisms.

The two types namely heterotrophic and autotrophic denitrification is commonly used for the treatment of municipal and industrial wastewater. However, increasing knowledge and experience indicates that biological treatment is the most promising, effective, and economic for removing nitrate from water and wastewater (Kim *et al.*, 2004). Biological denitrification is used to achieve the reduction of oxidized nitrogen compounds to gaseous nitrogen. As a result of the reduction, different intermediate and

end products are produced: nitrite (NO_2^-), nitric oxide (NO), nitrous oxide (N_2O) or molecular nitrogen (N_2). In the biological step of a wastewater treatment plant, the denitrification process to molecular nitrogen runs usually according to the following reaction (Chen and Lin, 1993; Shrimali and Singh, 2001).

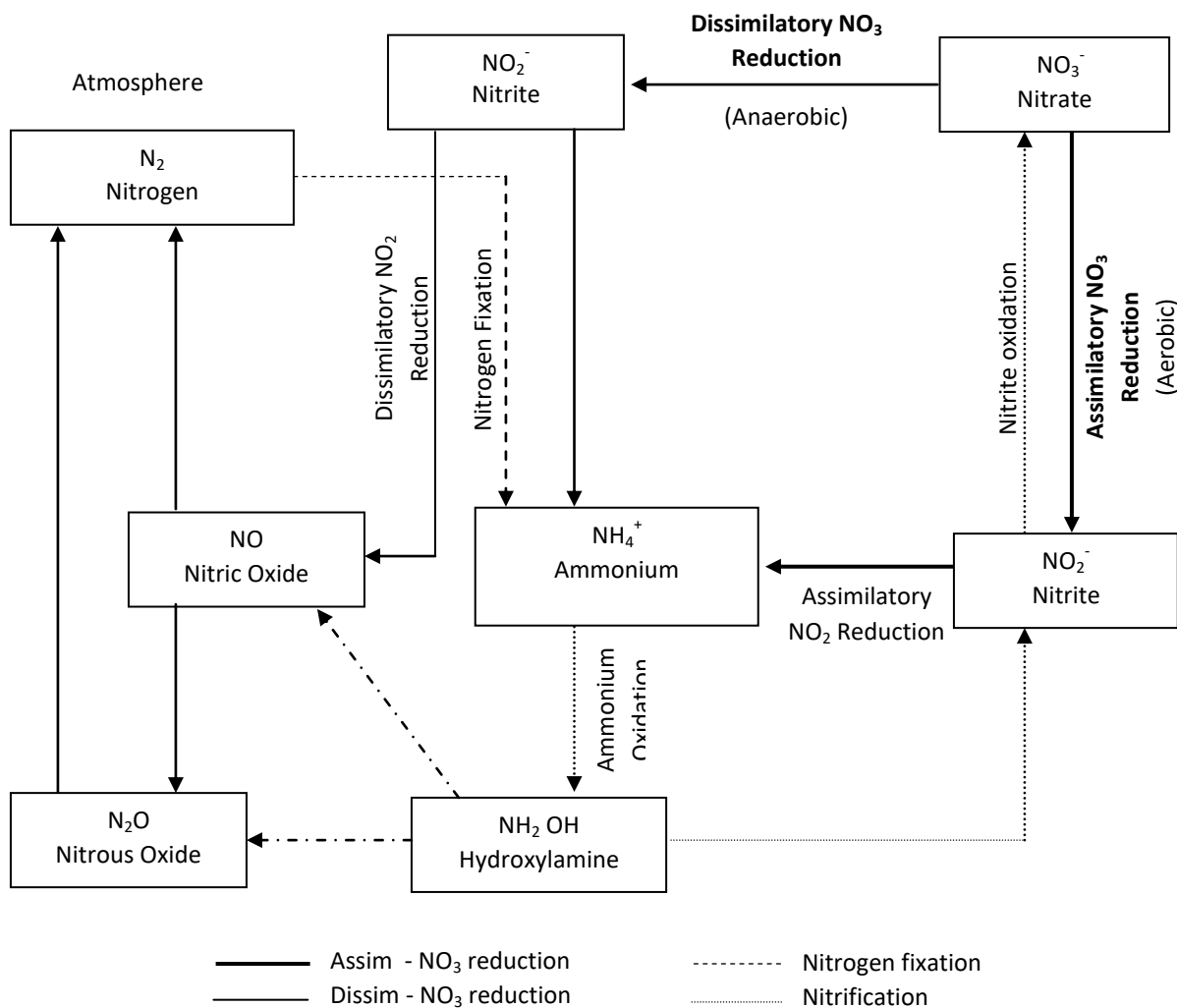


Fig. 2.1. Pathway of assimilatory and dissimilatory nitrate reduction

In nature riparian zones remove nitrate from groundwater and is believed to be due to microbial denitrification (Devito *et al.*, 2000). Denitrification requires a sufficient supply of nitrate as an energy source that is often organic matter under anaerobic conditions. These conditions are often met in riparian zones. Therefore, a system analogous to this has been proposed for remediation of contaminated groundwater and wastewater (Payne, 1993; Blaszczyk *et al.*, 1996; Schipper and Vukovic, 1998). Nitrogen

chemistry is complicated due to its numerous oxidation states in its compounds such as NO_3^- (+5), ammonium NH_4^+ (-3) and nitrite NO_2^- (+3) (Reddy and Lin, 2000).

Denitrification is generally accepted as the reduction process of nitrates, via nitrites, to nitrous oxides and nitrogen gas. However, many denitrifying bacteria have the enzymatic ability to reduce nitrates to nitrites only without further reduction of the nitrites produced (Cappuccino and Sherman, 1992; Robertson and Kuenen, 1992). Robertson and Kuenen (1992) reported that most of the denitrifying bacteria in aquatic systems are capable of only incomplete denitrification. However, the full effect of this on nitrogen removal during activated sludge treatment has not yet been determined (Drysdale *et al.*, 1999). Recently Fierro *et al.*, (2007) accomplished a study using an immobilized algal species such as *Scenedesmus* spp. for the removal of nitrate and found that 70% removal within 12 h of incubation.

Schipper and Vukovic (1998) created a porous reactive wall constructed in the polluted plume path to remove the contaminant as the groundwater passed through. Nitrate removal by denitrification is limited by the availability of an energy source. To overcome this, the reactive wall was constructed using 20% sawdust as a carbon source to stimulate denitrification. The wall successfully decreased nitrate concentrations in the groundwater throughout the study period. This removal could be due to denitrification or immobilization of nitrogen. However, immobilization seemed unlikely to be a major sink as the nitrogen removal in the wall, suggesting no net nitrogen accumulation. Denitrification over an extended time depends on nitrate input and a supply of carbon from degrading sawdust. It was predicted that overtime the sawdust would be degraded and nitrate removal would consequently decline. Therefore, fresh sawdust needs to be added annually. The main concern with biological denitrification is that there might be some remnant microbes in the treated water, which then requires post treatment. The major limitations of this process include the rate of substrate diffusion, reaction products through the matrix, loss of cells from the matrix, reduced activity of cells and short life span of the matrix (Kapoor and Viraraghavan, 1997). To overcome these problems, the biofilm reactor was invented, which keeps denitrifying bacteria and the carbon and energy source away from the treated water. The cost for a heterotrophic denitrification plant would be about twice that of ion

exchange with equivalent operating costs. The autotrophic biological system would cost approximately twelve times that of ion exchange (Kapoor and Viraraghavan, 1997).

Microbial denitrification is not unusual in soils with over 60% pore saturation where oxygen for microbial respiratory is limited (Dobbie & Smith, 2003). At present, the nitrate is eliminated both by physicochemical techniques that don't degrade it or through degradation by way of microorganisms that is a slow manner (Mellor *et al.* 1992). Microbial denitrification is an organic breathing procedure involving the successive reduction of nitrogen compounds: nitrate, nitrite, nitric oxide, and nitrous oxide, to nitrogen. The 4 steps are catalysed by means of four special enzymes (Pelmont, 2005). Interest in hydrogenotrophic denitrification for wastewater treatment has grown in recent a long time, Because of its low manufacturing of sludge (Rezania, 2005). In hydrogenotrophic cultures, denitrification and boom price are lower than in heterotrophic cultures.

Exclusive microorganisms own complete, partial or no denitrification activities, and can be more as it should be subdivided into five groups, complete denitrifiers (able to decreasing both nitrate and nitrite to N_2), unique nitrite reducers (best able to reducing nitrite however no longer nitrate to N_2), incomplete denitrifiers (reducing nitrate or nitrite to nitrogen oxide intermediates in place of N_2), incomplete nitrite reducers (capable of lowering nitrite to nitrogen oxide intermediates), and non-denitrifiers (not capable of nitrate or nitrite reduction). Examples of microorganisms falling into some of the above categories encompass *Hyphomicrobium* spp. (complete denitrifiers) (Sperl and Hoare, 1971). Important parameters that outline microbial network shape are the identity and abundance of the resident populations. Efforts to identify denitrifying microorganisms in various denitrifying ecosystems have historically depended on tradition-based totally techniques (Heylen *et al.* 2006). Denitrification functionality is enormous in both bacterial and archaeal domain names, and the range of denitrifying groups in other environments is typically better than those in wastewater remedy procedures (Throback, 2006). Nitrate discount to harmless nitrogen in unique absence oxygen situation is the governing principle of organic denitrification (BD) (Seidel, 2011).

4.3.5.2. Effect of carbon source on nitrate removal

Bacterial assimilation can be encouraged by providing readily degradable carbon source in a quantity commensurate with nitrate - N (Deshmukh, 1992). For

denitrification, readily biodegradable carbon source is mandatory (Bode *et al.*, 1987). It is suspected that poorly degradable carbon source results in nitrate-ammonification instead of nitrate denitrification. It is believed that reduction of nitrate into ammonium is favored in carbon - rich or nitrate - poor environments (Tiedje, 1981 and 1988).

A variety of carbon sources such as methanol (Gabaldon *et al.*, 2007), methane (Thalasso *et al.*, 1997), glycerol and sucrose (Pinar *et al.*, 1998), lactic acid (Akunna *et al.*, 1993), galactose, fructose and lactose (Pinar *et al.*, 1997), glucose, acetic acid and starch (Shanthi *et al.*, 2005), ethanol, acetate and sugars (Peng *et al.*, 2007) were used for nitrate removal from wastewater. Other authors worked on denitrification with ethanol (Blaszczyk, 1993) and acetic acid (Wilderer *et al.*, 1987) as carbon source, but the studies were carried out with an initial nitrate concentration lower than 10 mM. It was shown that ethanol and acetic acid allowed a very high denitrification rate and efficiency. The studies on the influence of two carbon sources (ethanol and acetic acid) for the denitrification rate of nitrate-concentrated industrial wastewater (115 mM nitrate) showed that high specific denitrification rate was observed on acetic acid and that acetic acid is a directly assimilable carbon source. Blaszczyk *et al.*, (1981) observed that acetic acid was found to be a good source of carbon for denitrification of both nitrate and nitrite. Both heterotrophic and autotrophic bacteria can carry out denitrification. Under anoxic conditions, heterotrophic bacteria utilize organic substrates including methanol, ethanol and acetic acid for the conversion of nitrate to nitrogen. In contrast, autotrophic denitrification utilizes substrates including hydrogen or reduced sulphur compounds while carbon dioxide or bicarbonate acts as the carbon source required for cell synthesis (Kapoor and Viraraghavan, 1997).

Akunna *et al.*, (1993) used batch test to determine the potentials of digested sludge to reduce nitrate and nitrite in the presence of five different carbon sources like glucose, glycerol, acetic acid, lactic acid and methanol. Ammonium accumulation was found in glucose and glycerol media. Dissimilatory reduction to ammonium accounted for up to 50% of reduced nitrate and nitrite. The rest was denitrified. Ammonification was higher than denitrification only when glucose and glycerol were present in the media. Several researchers made comparative studies on the effects of methanol and ethanol as external sources on denitrification (Hallin and Pell, 1998). Their studies

showed that ethanol should be a better external carbon source than methanol, especially when added to the denitrification zone of a single-sludge nitrogen removal system. In the latter case, the influent and external carbon is used concomitantly for denitrification and the external one is provided only when the ratio of influent COD to N is low.

Sreelatha *et al.*, (1997) isolated bacterial strains from activated sludge and *Pseudomonas* strain were examined denitrification process with respective carbon sources. The results showed that nitrite was accumulated under aerobic condition when nitrate and carbon sources were available. The carbon sources used were L - asparagine, sodium acetate, lactic acid, glucose, ethanol and methanol. Ideal carbon sources suitable for denitrification were L - asparagine and sodium acetate.

Methanol is the maximum typically employed outside carbon source because of being easily assimilated through denitrifying bacteria and its low cost (Tam 1992). Methanol: the use of each way of life-based and independent strategies, populations related to *Methylophilus*, *Paracoccus*, *Methyloversatilis* and *Hyphomicrobium* spp., have been recognized in numerous methanol-feeding denitrification structures (Claus and Kutzner 1985). With greater remedy plant life facing new stringent nutrient limits, the call for for outside carbon resources is anticipated to increase drastically, and its miles suited to apply carbon resources that have low cost, low biomass yield and high bloodless temperature kinetics. Many commercially to be had carbon assets can set off a better denitrification pastime than methanol (Bodík *et al.*, 2009).

Other carbon sources: Examined the shape of denitrifying populations below acetate- and complicated substrate mixture (acetate, ethanol and pyruvate)- feeding conditions (Morgan-Sagastume *et al.*, 2008). Even though the effects acquired with those carbon resources are very pleasant, the vital trouble while coping with a high nitrate awareness may be the accumulation of nitrites produced for the duration of denitrification, consequently inflicting inhibition of bacterial improvement (Glass, 1998). Nitrite detection was decided at 585 nm the use of the ferrous sulphate technique (HACH guide, tailored from McAlpine and Soule (McAlpine, 1933).

4.3.5.3. Effect of temperature on nitrate removal

The effect of temperature on denitrification rate is another important feature in the design of a denitrification process (Carrera, 2003). Generally, incubation near 30°C

is used to grow the microorganisms, but not always favors for denitrification. Thermophilic and psychrophilic denitrifying bacteria are known to have different optimum temperature from mesophiles (Konishi, 1969). High temperature reported is possibly a reflection of nitrobiological reactions (Bremmer and Shaw, 1958). Sreelatha *et al.* (1997) reported the effect of various factors such as pH, temperature and hardness on the rate of nitrate reduction and the level of nitrite production by *Pseudomonas* sp. Most of the studies on the effect of temperature were carried out in soil system. The rate of N₂O production was very high during denitrification at high temperature (60 - 70°C) (Keeney *et al.*, 1979) whereas at low temperature (0 - 5°C) the rate of denitrification decreased (Bailey and Beauchamp, 1973; Smid and Beauchamp, 1976). Ahsan *et al.*, (2005) studied the effect of temperature on the removal of suspended solid (SS), phosphate ion, nitrate ion, ammonium ion and chemical oxygen demand (COD) were evaluated using waste materials (refuse concrete and refuse waste) and natural material (shell). The results revealed that extensive improvement of removal efficiencies for nitrate ion and ammonium ion could not be achieved by increasing the temperature, the significant enhancement of the removal of SS and COD was observed.

Microbial boom rate in denitrification structures is influenced through diverse environmental conditions and physicochemical parameters including the availability of substrate, pH, temperature, alkalinity, C/N ratio, presence of competing ions, and other natural chemical compounds. Amongst these, temperature has been a considerable have an effect on on the microbial denitrification price. The excessive growth fee of denitrifying microorganism is associated with temperature boome (Liu *et al.* 2013). Hoover *et al.* (2016) The most excellent pH and temperature for wastewater denitrification are 7-9 and 20-30 °C respectively, with denitrification activity falling off unexpectedly out of doors of those stages (Grady *et al.* 1999).

3. MATERIALS AND METHODS

3.1. Bacterial strains

The bacterial strains GS1 to GS10 as nitrate reducers were obtained from Bioremediation Laboratory, Department of Microbiology, Periyar University, Salem, India. They were sub cultured in nutrient agar medium and maintained under aseptic conditions. The bacterial strains obtained were identified based on their morphology and biochemical characteristics as described by Bergey's manual of determinative bacteriology. The nutrient agar used in this study consists (g/l) 5g of sodium chloride, 5g of peptone, 3g of beef extract, 3g of yeast extract and 20 of agar and 1 lit of distilled water. The bacterial strains were sub cultured every 10 days for maintaining viability and preserved by using glycerol solution having a concentration of 20% (v/v), followed by stored at 4°C for further studies.

3.2. Media and chemicals

The bioremediation study for nitrate contamination was conducted in a mineral salt medium (MSM) (pH 8 ± 0.2) containing (g/l) 1.17g of NaCl, 0.30g of KCl, 0.15g of HN_4Cl , 0.41g of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.11g of CaCl_2 , 0.20g of KH_2PO_4 , 0.07g of Na_2SO_4 , 2g of NaHCO_3 and 0.4g of yeast extract. All analytical grade chemicals and reagents were purchased from Loba chemie (Loba Chemie Pvt, Ltd, Mumbai, 400005, India) and Hi-media laboratories (Hi-media laboratories, Mumbai, 400 086, India).

3.3. Identification of bacterial strains

The bacterial isolates were grouped to various genera based on their morphological and biochemical characters as given in Bergy's Manual of Determinative Bacteriology (Buchanan and Gibbons, 1974).

3.3.1. Gram staining

A loopful of overnight culture was taken and a uniform smear was made on a clean glass slide. It was heating fixed and then crystal violet solution was added onto the slide and kept for one minute. The slide was then washed with water and flooded with grams iodine solution, after one minute. The slide was washed and decolorized with 95% ethanol, then the slide was counter stained with Safranin for 30 seconds. The slide was then observed microscopically under 100x oil immersion and results were noted.

Crystal violet

Crystal violet	:	2.0 g in 20 ml of 95% ethanol Ammonium
oxalate	:	0.8 g in 80 ml of distilled water

Grams iodine

Iodine	:	1.0 g
Potassium iodide	:	2.0 g
Distilled water	:	300 ml

Safranin

Safranin	:	0.25 g
95% ethanol	:	10 ml
Distilled water	:	100 ml

3.3.2. Endospore staining

A loopful of overnight culture was taken and a smear was made on a clean glass slide. It was heat fixed; the slide was flooded with 0.5% malachite green and kept over a water bath for 5 minutes without boiling the stain. The slide was washed with water and then Safranin was added to the smear for 30 seconds. The slide was washed again, dried and observed under oil immersion and then results were noted.

Malachite green	:	5 g
Distilled water	:	100 ml
Safranin	:	0.5 %

3.3.3. Motility test

A sterile hanging drop slide was placed on the chamber with depression uppermost. A little Vaseline was spread around the cavity of the slide. Cover slip was cleaned and Vaseline was applied on each of the four corners of the cover slip using a tooth pick. The cover slip was placed on a clean paper with the Vaseline side up. A loopful of culture was transferred to the center of the cover slip. The depression slide was placed onto the cover slip with the cavity facing down and the slide was gently pressed to the form a seal between the cover slip and the slide. The hanging drop preparation was lift and turned quickly to suspend the culture drop. The preparation was examined microscopically and findings were recorded.

3.3.4. Oxidase test

All isolated strains were made as a smear onto an oxidase dry disc. Then observed findings were recorded.

3.3.5. Catalase test

A clean glass slide was taken and a drop of 3% hydrogen peroxide was added. The overnight culture was added to the drop and result were observed and recorded.

3.3.6. Indole test

The test culture was inoculated onto tryptone broth and incubated at 37°C for 24 to 48 hours. About 0.2 to 0.3 ml of Kovac's reagent was added to the test tubes and then tubes were shaken and allowed to stand. The formation of cherry red color ring on the surface of the broth confirmed the production of indole.

3.3.7. Methyl red test

A loopful of culture was inoculated into MR-VP broth. The tubes were incubated at 37°C for 24 h. The color change on addition of methyl red was noted.

3.3.8. Triple sugar ion test

A loopful of culture was stabbed and streaked on triple sugar ion (TSI) agar slant the tubes were incubated at 37°C for 24 to 48 hours.

3.4. Primary screening of nitrate reducing bacteria KNO₃ broth (g/L)

Peptone	:	5.0
Beef extract	:	3.0
Sodium chloride	:	5.0
Potassium nitrate	:	5.0
Distilled water	:	1000ml
pH	:	7.0 ± 0.2

The medium was prepared and sterilized for 15 minutes at 15 lb pressure. The bacterial isolates (GS1 to GS10) were inoculated and incubated at room temperature for three days. The ability of the isolates to reduce nitrate to nitrite and to ammonium was tested by the addition of 1ml of Nessler's reagent. The appearance of reddish orange colour showed that nitrate / nitrite has been reduced to ammonium. Based on the intensity of the colour (reddish orange), they were grouped into the following classification as dissimilatory nitrate reducing bacteria:

High	:	+++
Moderate	:	++
Low	:	+
No reaction	:	-

3.5. Antagonistic activity for making bacterial consortium

Muller Hinton agar plates were prepared and the plates were inoculated with the bacterial strains by surface swabbing. The plate was completely streaked with sterile swab containing GS1 bacterial culture and then four wells were punched with sterile well puncher. To each well, the other bacterial cultures (GS2 to GS10) were inoculated using sterile micropipette. Make sure that the culture does not overflow above the wells. Subsequently, the plate 2 to 10 were also inoculated in the same way with S2 to S10 bacterial cultures swabbed respectively and the wells filled with respective strains. All the plates were then incubated at 35°C for 24 hours and observed for any zone of inhibition around the wells. Based on the synergistic activity, the strain GS1 produces no antagonism with each other. Hence, GS1 was selected and used for further nitrate reduction in MSM.

3.6. Identification of potential strain GS1 through 16S rRNA sequencing

Molecular identification of the selected bacterial strain GS1 was analysed through 16S rRNA sequencing method. The genomic DNA of the potential strain was subcultured using a standard procedure. PCR amplification of 16S rRNA sequence from genomic DNA was performed using PCR protocol. The sequence thus obtained was compared using NCBI BLAST tool for similarity of bacterial species was interpreted (Ismail *et al.*, 2019). The sequences were aligned and phylogenetic tree was constructed for the potential strains using MEGA 6.01 software. From the 16S rRNA sequencing method, the bacterial strain GS1 was identified as *Priestia flexa* (GS1).

3.7. Nitrate removal from synthetic media

Different approaches were followed for the removal of nitrate from synthetic water samples contaminated with 100 mg/l of nitrate. First one was batch study, the second was reactor study and the third was fixed soil bed column study.

3.7.1. Batch study on nitrate reduction in synthetic medium

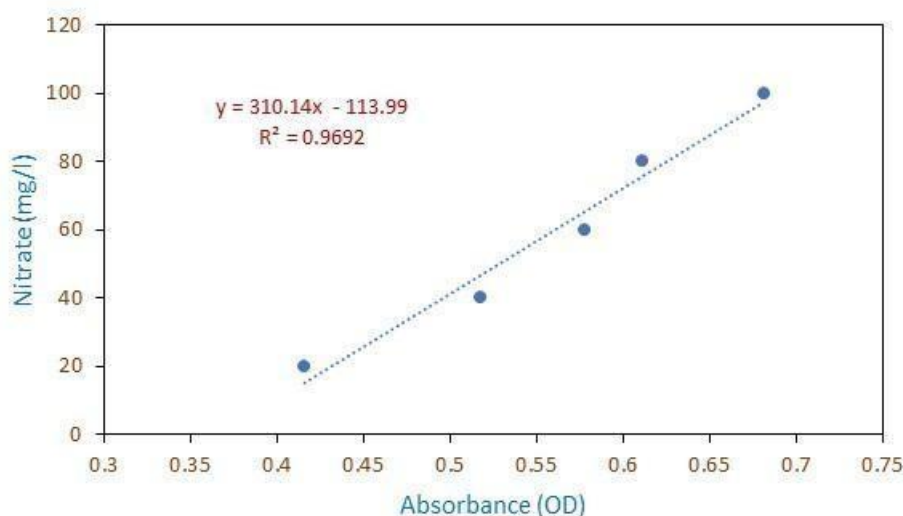
3.7.1.1. Preparation of inoculum

A loopful of culture of *Priestia flexa* (GS1) was inoculated in presterilized 100ml nutrient broth. The flask was kept in a shaker at 120 rpm for 12 hrs at 30°C. The culture broth was centrifuged at 4000 rpm for 20 min. Cell suspension was prepared using sterile distilled water and adjusted to 1OD (104 CFU/ml) using UV-Visible Spectrophotometer (Cyberlab UV-100, USA). One ml (104 CFU/ml) of the above suspension was used as inoculum for the nitrate reduction. This procedure was followed in entire studies.

3.7.2. Effect of various carbon sources on nitrate removal in synthetic medium

Mineral salts medium (MSM) was prepared with 100 mg/L of NO_3^- and supplemented with varying concentrations (0.5, 1.0 and 1.5%) of glucose, soluble starch and cellulose and sterilized. To this medium, about 1% of GS1 inoculum containing 10^4 CFU/ml of the cells were aseptically inoculated and kept in a shaker (120 rpm) at 30°C for 7 days (Plate 3.1). The sterilized synthetic medium without any carbon source was maintained as control to compare the effect of carbon source on nitrate removal by bacterial species. The samples were drawn aseptically at every 24h intervals (up to 7th day) and the growth (UV-Vis Spectrophotometric determination) and nitrate (Phenol disulphonic acid method) parameters were estimated (APHA, 2005). Estimation of nitrate was carried out using a standard way of phenol disulphonic acid method. Using this method, a standard graph was obtained by plotting the absorbance value Vs concentration of nitrate (Fig. 3.1). For the determination of nitrate in MSM, the absorbance (OD) was interpreted with the Y values ($y = 310.14x - 113.99$) of standard graph. Among different carbon sources used with varying concentrations, cellulose at 1% concentration exhibited a maximum degradation of nitrate. Hence, cellulose 1% was selected and used as sole source of carbon for further studies.

Fig. 3.1. A standard graph for determination of nitrate from MSM



3.7.3. Effect of natural organic substrate on nitrate removal in synthetic medium

MSM was prepared with 100 mg/l of NO_3^- and supplemented with 1% concentration of varying natural substrate namely, coir wastes, orange fruit residues, corn fruit silks and sugar cane bagasse and sterilized (Plate 3.2). To this medium, about 1% of GS1 inoculum containing 104 CFU/ml of the cells were aseptically inoculated and kept in a shaker (120 rpm) at 30°C for 7 days (Plate 3.1). The sterilized synthetic medium without any carbon source was maintained as control to compare the effect of natural substrate on nitrate removal by bacterium. The samples were drawn aseptically at every 24h intervals and tested for its nitrate concentrations.

3.7.4. Effect of various temperatures and pH's on nitrate removal in MSM

Since, the pH of the water samples and atmospheric conditions are varied from place to place, the effect of various temperatures (25, 30, 35, 40 and 45°C) and various pH (5, 6, 7, 8 and 9) on the removal of nitrate by GS1 was carried out in MSM supplemented with optimized cellulose concentration (1%) at various time intervals. Periodically the bacterial growth, nitrate, nitrite and ammonium concentrations were determined.

3.7.5. Removal of nitrate in synthetic water through a bioreactor approach

The removal of nitrate at 100 mg/l) from synthetic water was carried out through a bioreactor design and the setup made as shown the plate 3.3. This set up was

consisted bioreactor tank with agitation and aeration facilities. All the reactor tanks are 10L capacity and made up of tarson. In the bioreactors, about 10 litre of synthetically contaminated nitrate about 100 mg/l was taken and processed with bacterial strain of GS1 for 13 days. There are five different aspects like Treatment 1 to 5 was studied as shown in the following protocols:

Reactor 1: Sterile synthetic water + NO_3 (100 mg/l) + No Cellulose + GS1 (1%)

Reactor 2: Sterile synthetic water + NO_3 (100 mg/l) + Cellulose (0.5%) + GS1 (1%)

Reactor 2: Sterile synthetic water + NO_3 (100 mg/l) + Cellulose (0.75%) + GS1 (1%)

Reactor 2: Sterile synthetic water + NO_3 (100 mg/l) + Cellulose (1.0%) + GS1 (1%)

Reactor 2: Sterile synthetic water + NO_3 (100 mg/l) + Cellulose (1.25%) + GS1 (1%)

In each reactor containing synthetic nitrate water, the nitrate degrading bacterial strain GS5 was added aseptically with the addition of 1% starch as a sole carbon source. The control bioreactor served as addition of bacterial inoculum but the synthetic medium without cellulose as carbon source. After, addition of nutrients and inoculum, the nitrate (100 mg/l) spiked sample was subjected to agitation by impellers. The experiments were carried out at room temperature and every 24 hours of incubation the samples were collected aseptically by using sterile syringe. The samples were determined its bacterial growth, nitrate, nitrite and ammonium by standard methods.

Further, the samples were processed to remove microbial biomass and other solid materials though conventional coagulation process (Plate 3.4). From each reactor, about 1 litre bacterially treated samples were taken in beakers respectively. To this sample about 0.5 mg/l of lime was added and gently mixed using the overhead stirrer. The samples after processed were subjected sand filtration using the device as designed in the plate 3.5. The filtration tank was a cylindrical glass vessel having 30 cm in height and 10 cm in width. To this, large pebbles were packed in the bottom of the vessel followed by small pebbles, gravel, coarse sand, fine sand and activated carbon. The depth of the packing material was divided into 6 portions. About 1 litre samples from each reactor was filtered through this filter and analyzed it's nitrate, nitrite, ammonia and bacterial count using UV-Vis spectrophotometer (APHA, 2005).

3.7.6. Removal of nitrate in synthetic medium using lab scale soil column enriched with bacteria

Removal of nitrate at 100 mg/l from synthetic water was carried out through a column set up containing soil enriched with selected bacterial strain of GS1. The set up was made as shown in the plate 3.6. In the present study, the fertile soil was collected from vigorous area of Peryiar University campus and sieved to remove the presence of unwanted debris and larger stones. The processed soil was enriched with selected bacterial strain GS1 about 10⁴ CFU/g under aseptic condition using a hand sprayer. Further, the soil was made moisture with sterile distilled water and used for column study. The core columns made up of acrylic approximately 300 mm in length and 100 mm in diameter were used in this nitrate removal study. Prior to use, each column was sterilized 4-5 times with absolute alcohol (99.9%). Approximately, 3 kg of soil per column presumptively enriched with bacterial strains was taken and packed it compactly under aseptic conditions and closed tightly with holed caps. Column before fixing it in the stands was incubated at room temperature for 24 hrs to develop the well grown bacterial cells. There are four set up of soil column treatment from 1 to 4 was made and carried out at room temperature. To remove nitrate, an appropriate protocol was made and applied in each treatment column are as follows:

Column Treat 1: Soil alone (without C source and inoculum GS1)

Column Treat 2: Soil + Cellulose 1% + Inoculum 1% (GS1)

Column Treat 3: Soil + Cellulose 1% + Inoculum 1% (GS1)

Column Treat 4: Soil + Cellulose 1% + Inoculum 1% (GS1)

After incubation of each column at 24 h, the processed soil columns were and fixed in series of strands. The column with each hole was connected to silicon tubes. The tube from the top of the column was inserted into the treated water collection vessel and the tube from the bottom was connected to the reservoir containing 100 mg/l of synthetic nitrate water. The nitrate water from each flask was passed in to the soil column enriched with selected bacterial strains (down to up) with the help of peristaltic pump (Ravel, RH-P100VS-100-2H) for 30 days. Approximately, 10 ml/h was set as a flow rate to collect the leach out for the analysis of nitrate and other significant parameters. Every 24 h for 10 days, the treated water from each column was collected and processed for subsequent process.

Plate 3.1. Batch study on the removal of nitrate in mineral salts medium



Plate 3.2. Natural substrates used for nitrate removal in synthetic medium



Plate 3.3. Bioreactor study on the removal of nitrate in mineral salts medium



Plate 3.4. Coagulation treatment on the removal of nitrate and other solid matters in mineral salts medium

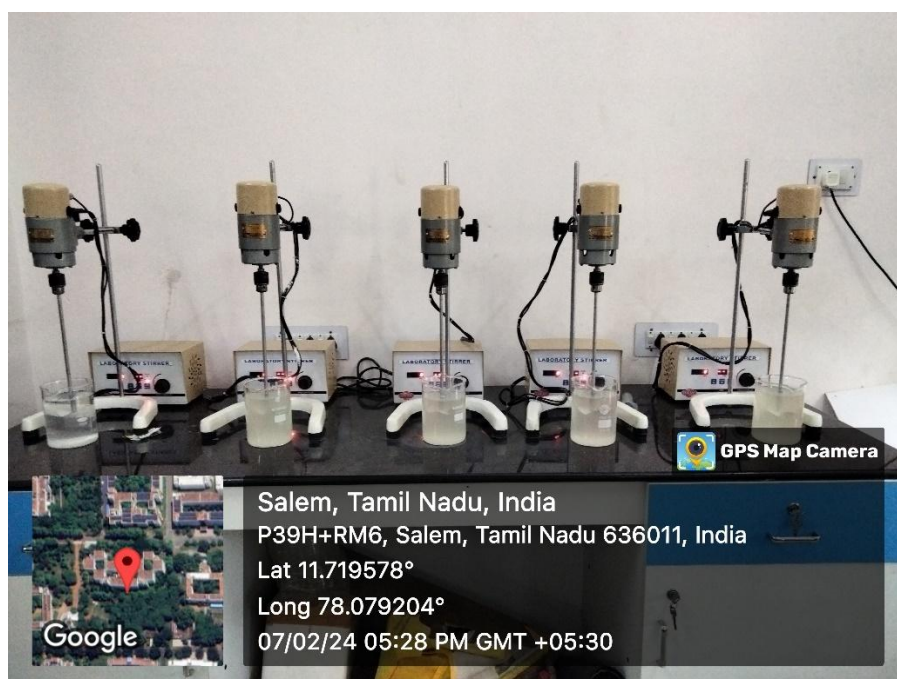


Plate 3.5. Sand filtration of treated water



Plate 3.6. Column study on the removal of nitrate from synthetic medium



4. RESULTS

4.1. Bacterial strains of nitrate reducers

Ten different bacterial strains that showed nitrate removal efficiency were collected from Bioremediation Research Laboratory, Department of Microbiology, Periyar University, Salem. The strains were purified by streaking then cultivated on nutrient agar slants which were labelled as GS1 to GS10 respectively and stored at 4°C for future studies.

4.2. Primary screening of nitrate reducing bacteria

The potassium nitrate broth medium was used in this study to find out its nitrate removal efficiency. The strains (GS1 to GS10) obtained were inoculated and incubated at room temperature for three days. The ability of the isolates to reduce nitrate to nitrite and to ammonium was tested by the addition of 1ml of Nessler's reagent. The appearance of reddish orange colour showed that nitrate/ nitrite has been reduced to ammonium. Based on the intensity of the colour (reddish orange), they were grouped into +++ as high nitrate reducers, ++ as moderate nitrate reducers and + as low nitrate reducers. However, all the strains obtained from the bioremediation laboratory showed higher efficiency (Table 4.1; Plate 4.1).

4.3. Antagonistic activity for making bacterial consortium

Potential strain GS1 was selected based on its synergistic activity against other Bacterial strains (GS2 to GS10). The strain GS1 showed no antagonism with each other and hence it was selected for further nitrate removal studies (Plate 4.2).

4.4. Identification of GS1 strain through 16S rRNA sequencing

The morphological and cultural characteristics of the potential bacterial strain GS1 was observed initially. While observing the preliminary identification results, it was evident that the strain belonged to the genus *Priestia* sp. Later, the molecular identification of the strains through 16S rRNA sequencing showed that the sequence results of GS1 with greater base pairs was similar to *Priestia flexa* (PP275113). The evolutionary tree for GS1 was constructed using MEGA 6.01 software (Plate 4.2).

4.5. Effect of carbon sources on the bacterial growth (GS1), nitrate removal, nitrite and ammonium formation in synthetic medium

The effect of the carbon sources like glucose, starch and cellulose at various concentrations (0.5%, 1.0% and 1.5%) were tested on nitrate reduction using *Priestia flexa* (GS1). Simultaneously the bacterial growth, nitrite and ammonium formation were also estimated. Mineral salts medium incorporated with 100 mg/l of

nitrate and varying carbon sources was used in this study in order to find out its nitrate removal efficiency. Among 10 bacterial strains GS1 was selected based on its nitrate reduction efficiency and physical nature of the strains. For nitrate removal in MSM, about 1 ml of bacterial strains GS1 containing 10^4 CFU/ml of the cells were used as inoculum. The sterilized MSM without any carbon source was maintained as control to compare the effect of carbon source on nitrate removal by bacterial species. The samples were drawn aseptically at every 24 h intervals (up to 8th day) and the growth and removal of nitrate were estimated. The results of this study were interpreted in figures 4.1 to 4.3. The results of the effect of carbon source on the above said parameters are given below.

4.5.1. Effect of glucose on the growth of GS1

The growth of the GS1 in synthetic medium with different concentration of glucose (0.5%, 1.0% and 1.5%) containing nitrate is shown in figure 4.1A. The growth of GS1 was maximum in 1% concentration of glucose in synthetic medium supplemented with glucose (100 mg/l) in entire incubation period. The growth was maximum which was 0.627 OD after 6th day of incubation. The growth was slightly less in the MSM with glucose at 0.5 and 1.5% respectively.

4.5.2. Effect of glucose on nitrate reduction in synthetic medium

The reduction of nitrate content in the synthetic medium containing various concentration of glucose (0.5%, 1.0% and 1.5%) by GS1 is given in figure 4.1B. The GS1 reduced nitrate from 100 to 28.13 mg.l⁻¹ in synthetic medium with 0.5% glucose on 4th day of incubation. High nitrate reduction was noticed in 0.5% glucose supplemented synthetic medium compared to 1.0% and 1.5%. Reduction of nitrate in 2 % concentration of glucose was from 100 to 27.16 mg.l⁻¹ on 2nd day of the experiment and thereafter slightly increased. The nitrate reduction at 1% glucose showed very negligible than 0.5 and 1.5% concentrations of glucose.

4.5.3. Formation of nitrite in synthetic medium supplemented with glucose

Nitrite formation in synthetic medium supplemented with various concentration of glucose (0.5%, 1.0% and 1.5%) and inoculated with GS1 is given in figure 4.1C. The nitrite formation during nitrate reduction was high (2.9 mg/l) in synthetic medium amended with 1.5% glucose on 2nd day of the experiment. It was gradually decreased and got 0 mg/l after 5th day of the study. The nitrite level in the medium with 0.5 and 1.0% concentration of glucose showed very negligible. After 4th

day there was no nitrite formation was recorded in the medium with each concentration of glucose.

4.5.4. Formation of NH_4 in synthetic medium supplemented with glucose

The formation of ammonium due to nitrate removal by GS1 supplemented with various concentration of glucose (0.5%, 1.0% and 1.5%) in synthetic medium containing 100 mg/l of nitrate is shown in figure 4.1D. The amount of ammonium formed during nitrate reduction varied with glucose at various concentrations. The amount of ammonium formed was maximum (30.96 mg/l) in MSM supplemented with 1.5% glucose and 100 mg.l⁻¹ of nitrate. It was stagnant until the incubation period of 7th day. The amount of ammonium was shown maximum (30.81 mg/l) in the medium with 1% glucose on the 1st day and thereafter gradually decreased. There was a significant difference in the formation of ammonium by the GS1 in the medium with 0.5% glucose. It got rapidly down after 24 h incubation of the medium.

4.6. Effect of starch on the bacterial growth (GS1), nitrate removal, nitrite and ammonium formation in synthetic medium

The effect of the starch at various concentrations (0.5%, 1.0% and 1.5%) was tested on nitrate reduction. Simultaneously the bacterial growth, nitrite and ammonium formation were also estimated. The results of this study were interpreted in figures 4.2. The results of the effect of carbon source on the above said parameters are given below.

4.6.1. Effect of starch on the growth of GS1

During nitrate reduction, the growth of the GS1 in synthetic medium with different concentration of starch (0.5%, 1.0% and 1.5%) is shown in figure 4.2A. The growth of GS1 was maximum in 1.5% concentration of starch in synthetic medium supplemented with 100 mg/l in entire incubation period. The growth was maximum which as 1.61 OD after 2nd day of the incubation period. The growth was slightly less in the medium with starch at 0.5 and 1.0% respectively.

4.6.2. Effect of starch on nitrate reduction in synthetic medium

Similar to glucose, the reduction of nitrate content in the synthetic medium containing various concentration of starch (0.5%, 1.0% and 1.5%) by GS1 was studied and the results are given in figure 4.2B. The GS1 reduced nitrate from 100 to 21.95 mg.l⁻¹ in synthetic medium with 1% starch on 4th day of the incubation. It was slightly increased after 4th day of the incubation period. The medium with 0.5% starch stands high nitrate reduction next to 1% concentration which was 100 to 41.73

mg/l. Starch at 1.5% concentration shown there was negligible reduction of nitrate. However, the concentration of nitrate recorded below the permissible limits.

4.6.3. Formation of nitrite in synthetic medium supplemented with starch

During nitrate reduction by the bacterial strain GS1, the formation of nitrite was tested in synthetic medium supplemented with various concentration of starch (0.5%, 1.0% and 1.5%) and the results are given in figure 4.2C. The nitrite formation during nitrate reduction was very high (3.71 mg/l) in synthetic medium amended with 1.5% starch on 2nd day of the experiment. It was suddenly decreased to 0.24 mg/l after 3rd day of the study. The nitrite level in the medium with 0.5 and 1.0% concentration of starch showed very insignificant. After 3rd day there was slight formation of nitrite was recorded in the medium with each concentration of starch.

4.6.4. Formation of NH_4 in synthetic medium supplemented with starch

The formation of ammonium due to nitrate removal by GS1 supplemented with various concentration of starch (0.5%, 1.0% and 1.5%) in synthetic medium containing 100 mg/l of nitrate is shown in figure 4.2D. The amount of ammonium formed during nitrate reduction varied with starch at various concentrations. The amount of ammonium formed was maximum (30.81 mg/l) in MSM supplemented with 1% starch and 100 mg.l⁻¹ of nitrate. Thereafter the ammonium content was got decreased. It was insignificant until the incubation period of 7th day. The amount of ammonium was shown maximum (30.96 mg/l) in the medium with 1.5% starch on the 3rd day and thereafter constant until 7 days incubation. There was a significant difference in the formation of ammonium by the GS1 in the medium with 0.5% starch. It got hastily down after 2nd day of the incubation period.

4.7. Effect of cellulose on the bacterial growth (GS1), nitrate removal, nitrite and ammonium formation in synthetic medium

The effect of the cellulose at various concentrations (0.5%, 1.0% and 1.5%) was tested on nitrate reduction. Simultaneously the bacterial growth, nitrite and ammonium formation were also estimated. The results of this study were interpreted in figures 4.3. The results of the effect of cellulose at different concentrations on the above said parameters are given below.

4.7.1. Effect of cellulose on the growth of GS1

The growth of the GS1 in synthetic medium with different concentration of cellulose (0.5%, 1.0% and 1.5%) containing nitrate is shown in figure 4.3A. The growth of GS1 was maximum in 1.5% concentration of cellulose in synthetic medium

supplemented with nitrate in entire incubation period. The growth was maximum which as 2.48 OD on 1st day of incubation period. Thereafter the growth was stagnant until 7th day of incubation. The growth was slightly less in the synthetic medium with cellulose at 0.5 and 1% respectively.

4.7.2. Effect of cellulose on nitrate reduction in synthetic medium

The reduction of nitrate content in the synthetic medium containing various concentrations (0.5%, 1.0% and 1.5%) of cellulose by GS1 is given in figure 4.3B. The GS1 reduced nitrate from 100 to 9 mg.l⁻¹ in synthetic medium with 1% cellulose on 2nd day of incubation. Afterwords, there was a slight reduction of nitrate recorded. High nitrate reduction was also noticed in 0.5% and 1% concentrations of cellulose in the medium after 2nd day of incubation period. The nitrate reduction was almost constant after 2nd day of incubation in the medium with each concentration of cellulose.

4.7.3. Formation of nitrite in synthetic medium supplemented with cellulose

Nitrite formation in synthetic medium supplemented with various concentration of cellulose (0.5%, 1.0% and 1.5%) by the bacterial strain GS1 was tested and the results are given in figure 4.3C. The nitrite formation during nitrate reduction was high (4.5 mg/l) in synthetic medium amended with 1% concentration of cellulose on 2nd day of the experiment. It was gradually decreased and got 0 mg/l after 6th day of the study. The nitrite level in the medium with 0.5 was very less. The formation of nitrite at 1.0% concentration of cellulose showed very negligible. After 2nd day, there was slight formation of nitrite was recorded in the medium with 1.5% concentration of cellulose.

4.7.4. Formation of NH₄ in synthetic medium supplemented with cellulose

The formation of ammonium due to nitrate removal by GS1 in the synthetic medium supplemented with various concentration of cellulose (0.5%, 1.0% and 1.5%) was tested and the results are shown in figure 4.3D. The amount of ammonium formed during nitrate reduction was varied with cellulose at various concentrations. The amount of ammonium formed was maximum (11.90 mg/l) in MSM supplemented with 1% cellulose and 100 mg.l⁻¹ of nitrate. It was decreased after 1st day of incubation. The amount of ammonium was shown 9.45 mg/l in the medium with 1.5% cellulose on the 1st day and thereafter slight decrease and constant. There was a significant difference in the formation of ammonium by the GS1 in the medium with 0.5% cellulose. It got constant after 1st day of incubation of the medium.

3.7.4. Effect of natural organic substrate on nitrate removal in synthetic medium

Synthetic MSM was used with 100 mg/l of NO_3^- and supplemented with 1% concentration of varying natural substrate namely, coir wastes, orange fruit residues, corn fruit silks and sugar cane bagasse and sterilized. Nitrate removal was studied in this medium with 1% of GS1 inoculum containing 10^4 CFU/ml of the cells were aseptically inoculated and kept in a shaker (120 rpm) at 30°C for 7 days. The results revealed that nitrate removal efficiency showed very negligible when natural substances used.

4.8. Nitrate removal in MSM at various temperatures

The effect of various temperatures (25, 30, 35, 40 and 45°C) on the removal of nitrate by the strain GS1 was carried out in MSM supplemented with optimized cellulose concentration of 1% at various time intervals (Fig. 4.4). This study was carried out since the temperature of the area and atmospheric conditions are varied from place to place. This was another batch study carried out for 7 days and the results were interpreted. Among the study with different temperatures, 35°C exhibits maximum removal of nitrate. At the 35°C, the nitrate reduction was recorded from 100 to 31.88 mg/l on 3rd day of incubation period. Thereafter, the nitrate level was constant and showed only a little bit difference. This might be due to supports of suitable temperature for the removal of nitrate. The temperature 30°C provided a significant reduction of nitrate. During nitrate reduction the growth of the bacterium was tested. The growth was showed maximum in 25°C followed by 30 and 35°C.

Nitrite formation in synthetic medium supplemented with optimized cellulose at 1% by the bacterial strain GS1 was tested and the results are given. The incubation temperature 25°C provided high nitrite formation (3.56 mg/l) during nitrate reduction in synthetic medium amended with 1% concentration of cellulose on 1st day of the experiment. It was gradually decreased till 4th day and thereafter showed constant. Only slight difference of nitrite formation recorded in the medium with 30 and 35°C. The formation of ammonium in the synthetic medium supplemented with 1% cellulose (0.5%, 1.0% and 1.5%) was tested. The amount of ammonium formed during nitrate reduction was varied with cellulose at various concentrations. The amount of ammonium formed was maximum (6.66 mg/l) in MSM incubated at 25°C. A gradual increase of ammonium was noted in medium incubated at 30°C. There

was a significant difference in the formation of ammonium by the GS1 in the medium with incubation temperature on 35°C.

4.9. Nitrate removal in MSM at various pHs

Since, the pH of the water samples is varied from place to place, the effect of various pH's (5, 6, 7, 8 and 9) on the removal of nitrate by GS1 was carried out in MSM supplemented with optimized cellulose concentration of 1% at varying time intervals (Fig. 4.5). Periodically the bacterial growth and nitrate was determined. The growth of the GS1 bacterium shown almost same in all pHs. However, the reduction of nitrate was significant and showed variation in the medium with varying pHs. Among the study carried out with different pH's, pH7 exhibits maximum removal of nitrate. This might be due to supports of hydrogen ion concentration for the removal of nitrate and also the growth of the bacterium. Nitrite formation in synthetic medium supplemented with 1% cellulose by the bacterial strain GS1 was tested. The nitrite formation during nitrate reduction was very trace level in the medium with all pHs. The amount of ammonium formed during nitrate reduction was varied in the medium with varying pHs. The amount of ammonium formed was maximum which was 10.44 mg/l in MSM with pH6 on 1st day of incubation. In further incubation period, the amount of ammonium was decreased gradually. There are no much more variations of ammonium formation was noted in the study carried out with other pHs.

4.10. Removal of nitrate in MSM through a bioreactor approach

Bioreactor study was carried out for the removal of nitrate (100 mg/l) from synthetic MSM and the results was given in the figure 4.6. Each reactor containing 100 mg/l of nitrate water was treated with the bacterial strain of GS1 under aerobic condition. The experiments were carried out at room temperature for 13 days and every 24 hours of incubation the samples were collected aseptically by using sterile syringe and tested its bacterial growth, nitrate reduction, formation of nitrite and ammonium. In order to find out the efficiency of nitrate removal by GS1, different concentrations (0.5, 0.75, 1.0 and 1.25%) of cellulose in the synthetic medium was used. A control reactor with aqueous medium without cellulose was maintained.

The maximum removal of nitrate was recorded in the bioreactor containing MSM supplemented with 1% concentrations of cellulose. The nitrate reduction was higher (from to 100 to 8.19 mg/l) on 6th day of treatment. After 6th day there was a slight increasing of nitrate recorded. There is no much difference on nitrate removal was recorded in other concentrations of cellulose. The nitrate removal was very

negligible in control bioreactor. Hence, it could be accomplished that, the bioreactor approach can be a suitable eco-friendly technique for the removal of nitrate.

The growth of the GS1 bacterium was recorded which was maximum in 1.25% concentration of cellulose. In the medium with other concentrations of cellulose, the growth of the bacterium showed insignificant. Similarly, the formation of nitrite and ammonium was tested during the reduction of nitrate in the synthetic medium treated with bioreactors. The formation of nitrite showed higher (18 mg/l) in control reactor containing synthetic medium with no carbon source. The formation of nitrite was very trace in the bioreactor 2 to 5. The formation of ammonium was higher in the medium supplemented with 1% cellulose on 2nd day of incubation. After 2nd day, the ammonium content was gradually decreased. Almost similar efficiency of ammonium formation was noted in other bioreactors from 2 to 5.

4.11. Removal of nitrate and other solid matters using inorganic coagulants

The bacterially treated samples from each bioreactor were processed to remove microbial biomass and other solid materials through conventional coagulation process. From each reactor, about 1 litre bacterially treated samples were taken in beakers respectively. To this sample about 0.5 mg/l of lime was added as coagulating agent and gently mixed using the overhead stirrer. The samples after processed were subjected sand filtration using the sand filter device. The samples after filtration were analyzed its nitrate, nitrite, ammonia and bacterial count using UV-Vis spectrophotometer. The amount of nitrate, nitrite, ammonia and bacterial count were very trace level and attained its treatment significantly.

4.12. Removal of nitrate in MSM through a fixed bed column approach

The bacterial strain GS1 was used in this study in order to find out its nitrate removal efficiency through a fixed bed soil column (Fig. 4.7). Removal of nitrate at a concentration of 100 mg/l was carried out through a column set up containing soil enriched with strain GS1. After the column set up made, the synthetic water containing 100 mg/l of nitrate was passed in to the soil columns (treatment 1 to 4) enriched with selected bacterial strain of GS1 (down to up) with the help of peristaltic pump (Ravel, RH-P100VS-100-2H) for 10 days. Approximately 10 ml/h was set as a flow rate to collect the leach out for the analysis of nitrate and other significant parameters. Every day of the study for 10 days, the treated water from each column was collected and processed for subsequent process.

Among 4 different treatments studied, treatment 4 (soil with 1.5% cellulose and 1% inoculum) showed significant removal of nitrate from 100 to 37.91 mg/l on 1st day. Thereafter there was a gradual removal of nitrate. The study carried out with treatment 2 to 3 shown somewhat negligible removal of nitrate. This might be due to varying circumstances namely, environmental factors like temperatures, pH and room humidity. Formation of nitrite shown significant in the column treatment 1. There is no nitrite formation recorded in treatment columns from 2 to 4. In each column treatment the formation of ammonium showed a gradual increase. However, the treatment column 4 containing soil and 1.5% concentration of cellulose shown maximum production of ammonium.

Table 4.1. Screening of nitrate reducing bacterial strains obtained from bioremediation laboratory, Periyar University, Salem

S.No	Strain Code	Genera	Nitrate Reduction
1	GS1	<i>Priestia flexa</i>	+++
2	GS2	<i>Bacillus</i> sp.	++
3	GS3	<i>Alcaligenes</i> sp.	++
4	GS4	<i>Corynebacterium</i> sp.	+
5	GS5	<i>Bacillus</i> sp.	++
6	GS6	<i>Bacillus</i> sp.	++
7	GS7	<i>Bacillus</i> sp.	++
8	GS8	<i>Corynebacterium</i> sp.	++
9	GS9	<i>Alcaligenes</i> sp.	+
10	GS10	<i>Bacillus</i> sp.	++

A photograph of six test tubes held in a white plastic rack. The tubes are arranged in a single row. From left to right, they are labeled: 'S5', 'GS4', 'GS3', 'GS2', 'GS1', and 'CONTROL'. Each tube contains a liquid that shows a color gradient from yellow to orange. The 'CONTROL' tube on the far right contains a clear, colorless liquid. The tubes are capped with white cotton wool. The rack is placed on a dark surface.

Phylogenetic tree showing the relationship between *Priestia flexa* and other species. The tree is rooted at the bottom with *Peribacillus huizhouensis*. The top branch is *Priestia flexa* GS1 PP275113, which is highlighted with a red box. Other branches include *Priestia flexa* IFO15715 NR 024691, *Priestia paraflexa* RC2 NR 135732, *Priestia flexa* NBRC 15715 NR 113800, *Priestia megaterium* ATCC 14581 NR 117473, *Priestia aryabhattai* B8W22 B8W22 NR 115953, and *Priestia qingshengii* G19 NR 133978.

Fig. 4.1. Effect of glucose at various concentrations on the removal of nitrate in synthetic medium (A) Bacterial Growth (B) Nitrate reduction (C) Nitrite Formation and (D) Ammonium Formation

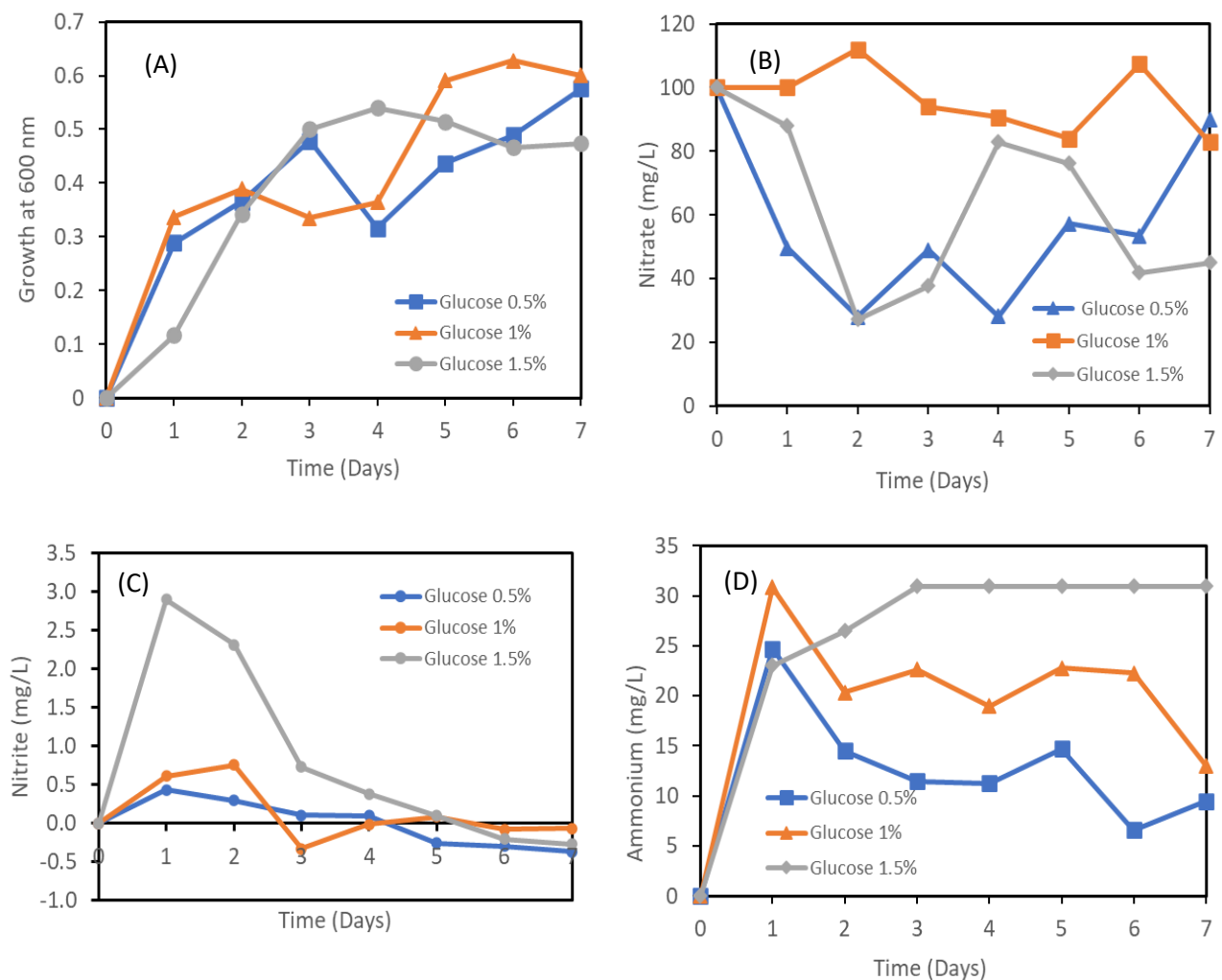


Fig. 4.2. Effect of starch at various concentrations on the removal of nitrate in synthetic medium (A) Bacterial Growth (B) Nitrate reduction (C) Nitrite Formation and (D) Ammonium Formation

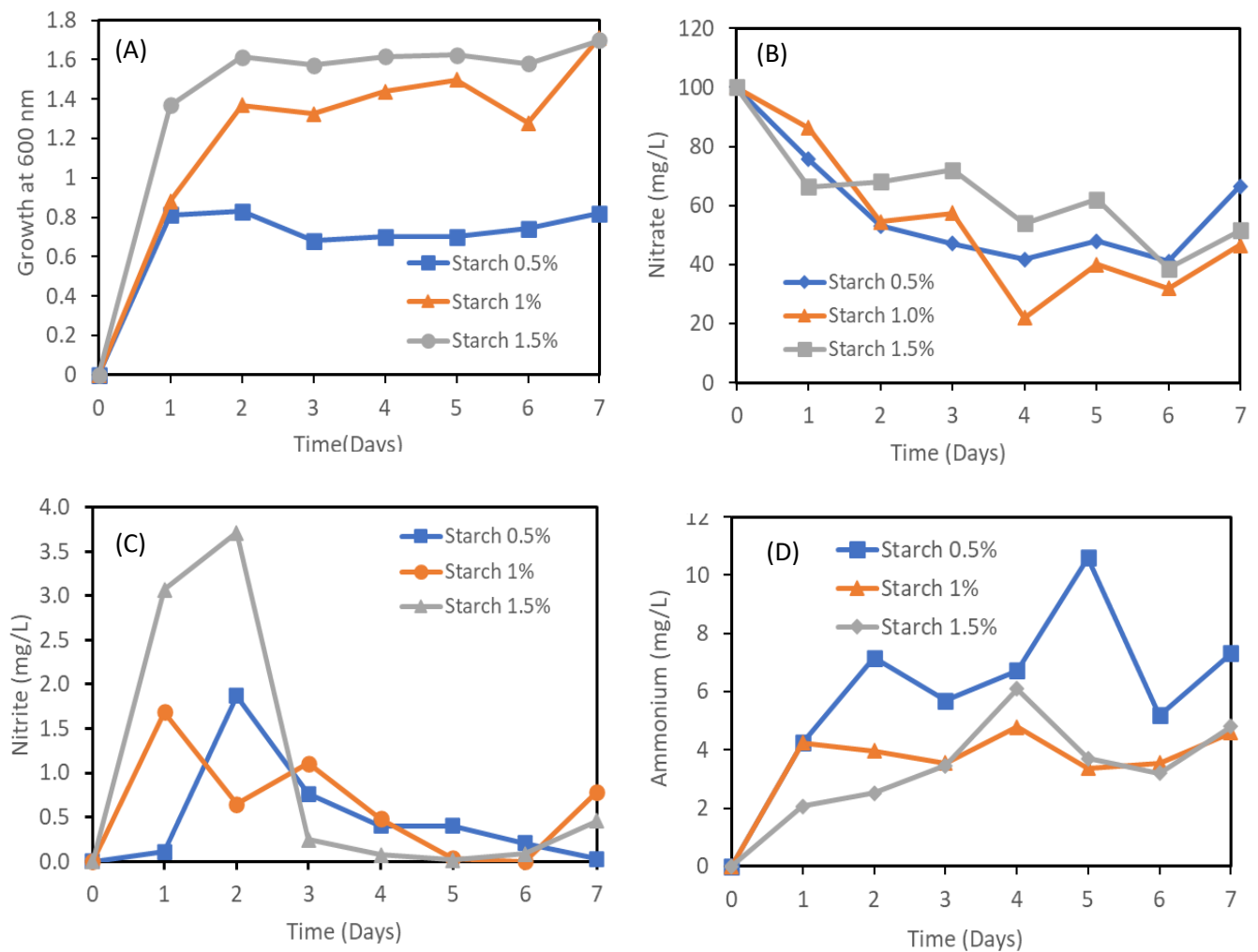


Fig. 4.3. Effect of cellulose at various concentrations on the removal of nitrate in synthetic medium (A) Bacterial Growth (B) Nitrate reduction (C) Nitrite Formation and (D) Ammonium Formation

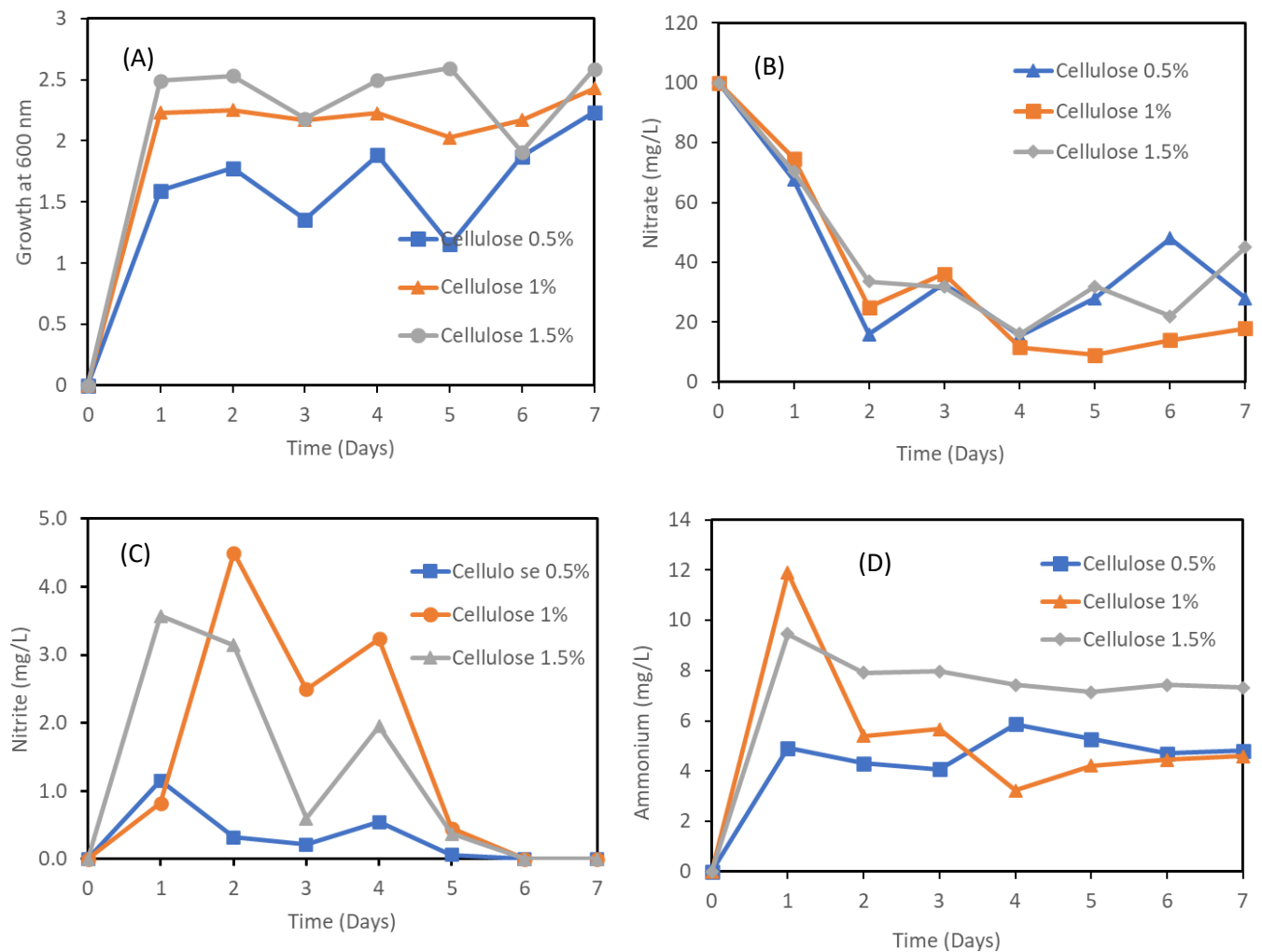


Fig. 4.4. Effect of various temperatures on the removal of nitrate in synthetic medium (A) Bacterial Growth (B) Nitrate reduction (C) Nitrite Formation and (D) Ammonium Formation

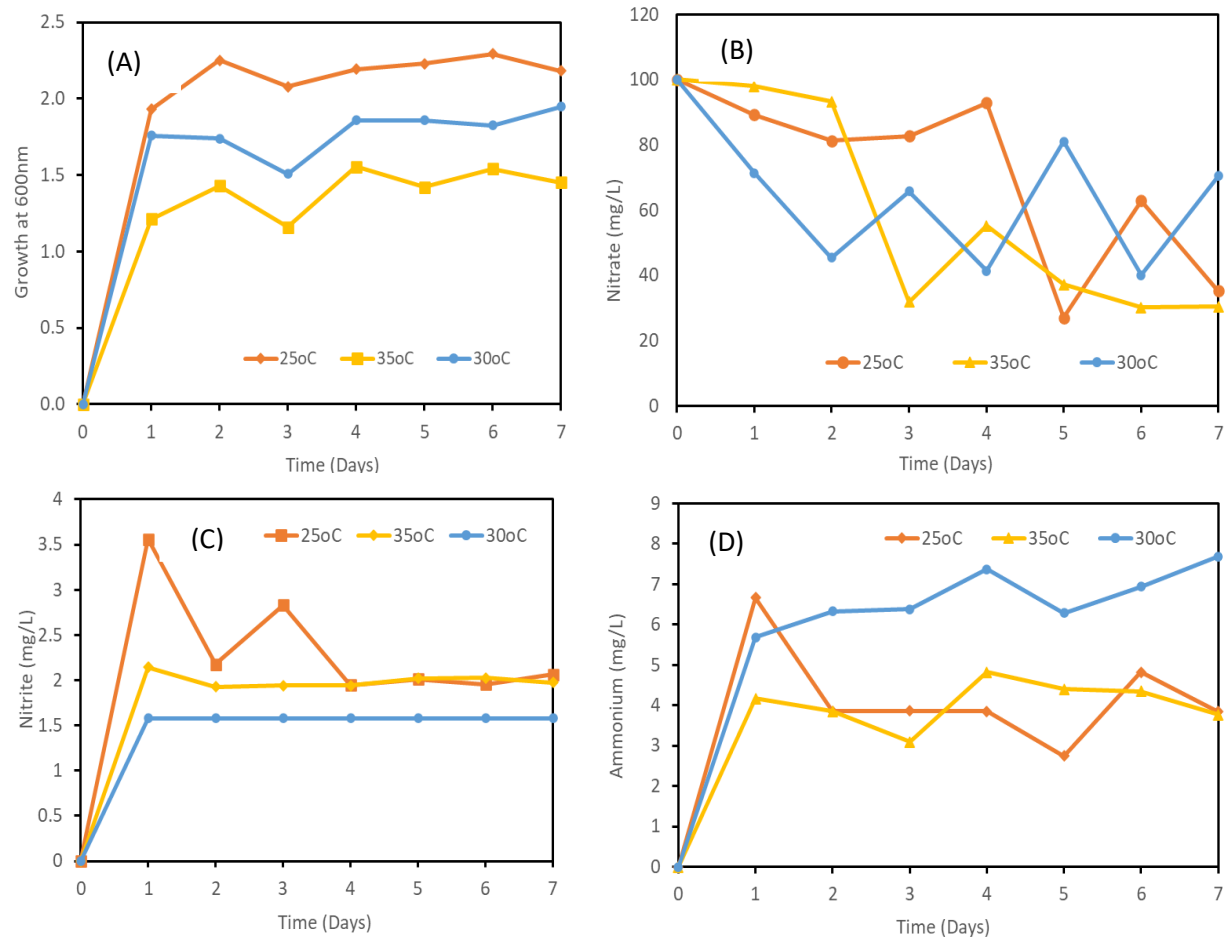


Fig. 4.5. Effect of various pH on the removal of nitrate in synthetic medium (A) Bacterial Growth (B) Nitrate reduction (C) Nitrite Formation and (D) Ammonium Formation

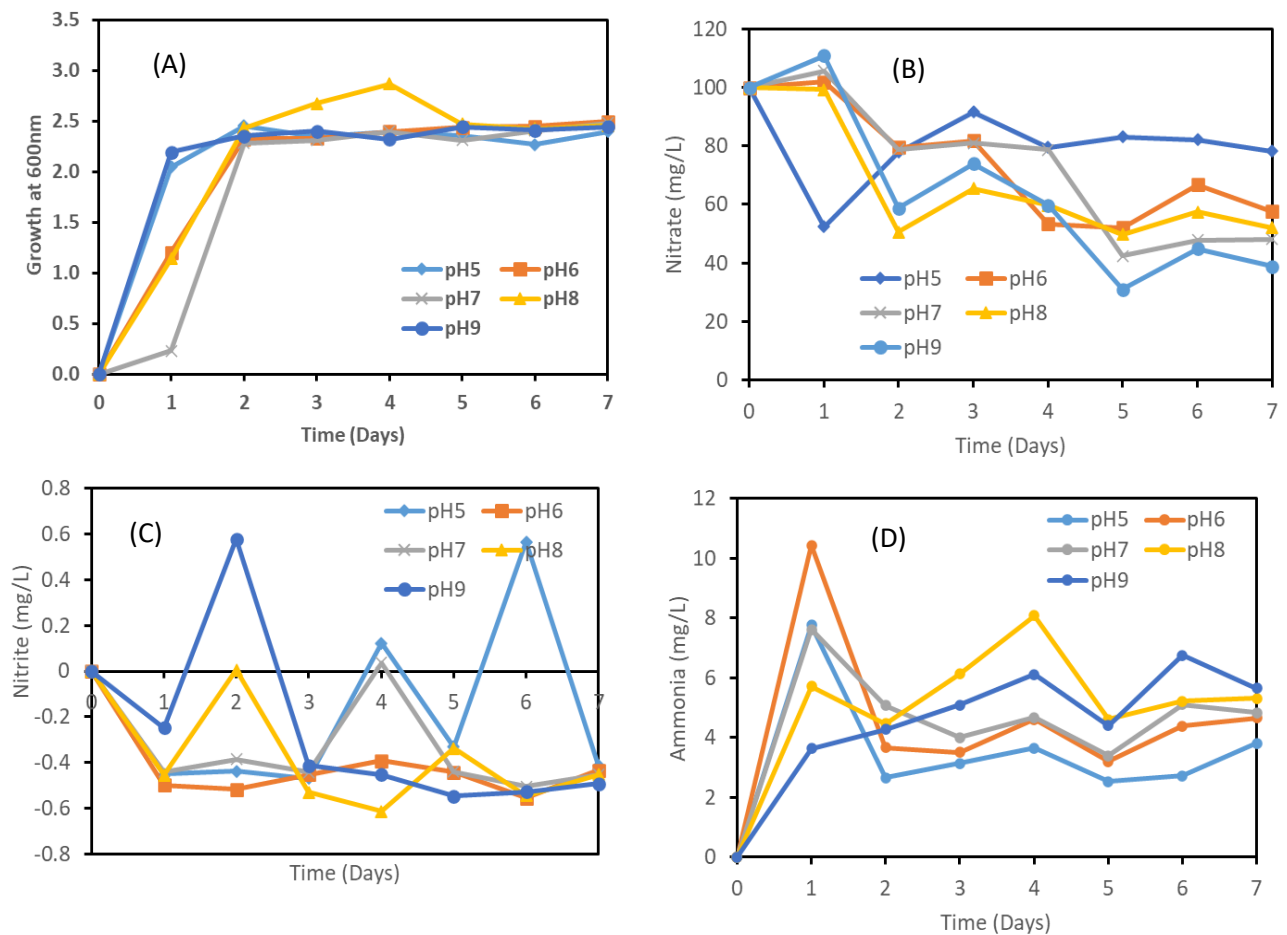


Fig. 4.6. Removal of nitrate in MSM through a bioreactor approach (A) Bacterial Growth (B) Nitrate reduction (C) Nitrite Formation and (D) Ammonium Formation

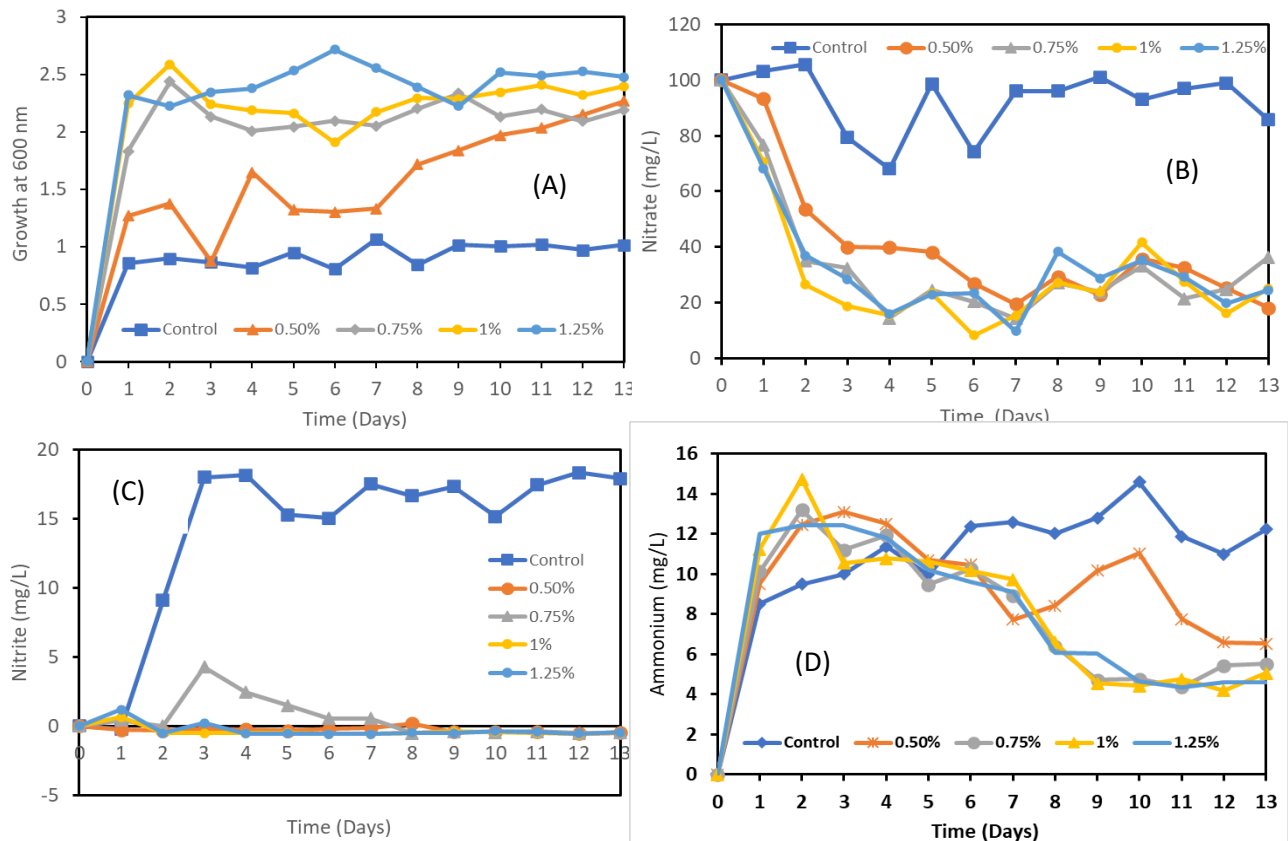
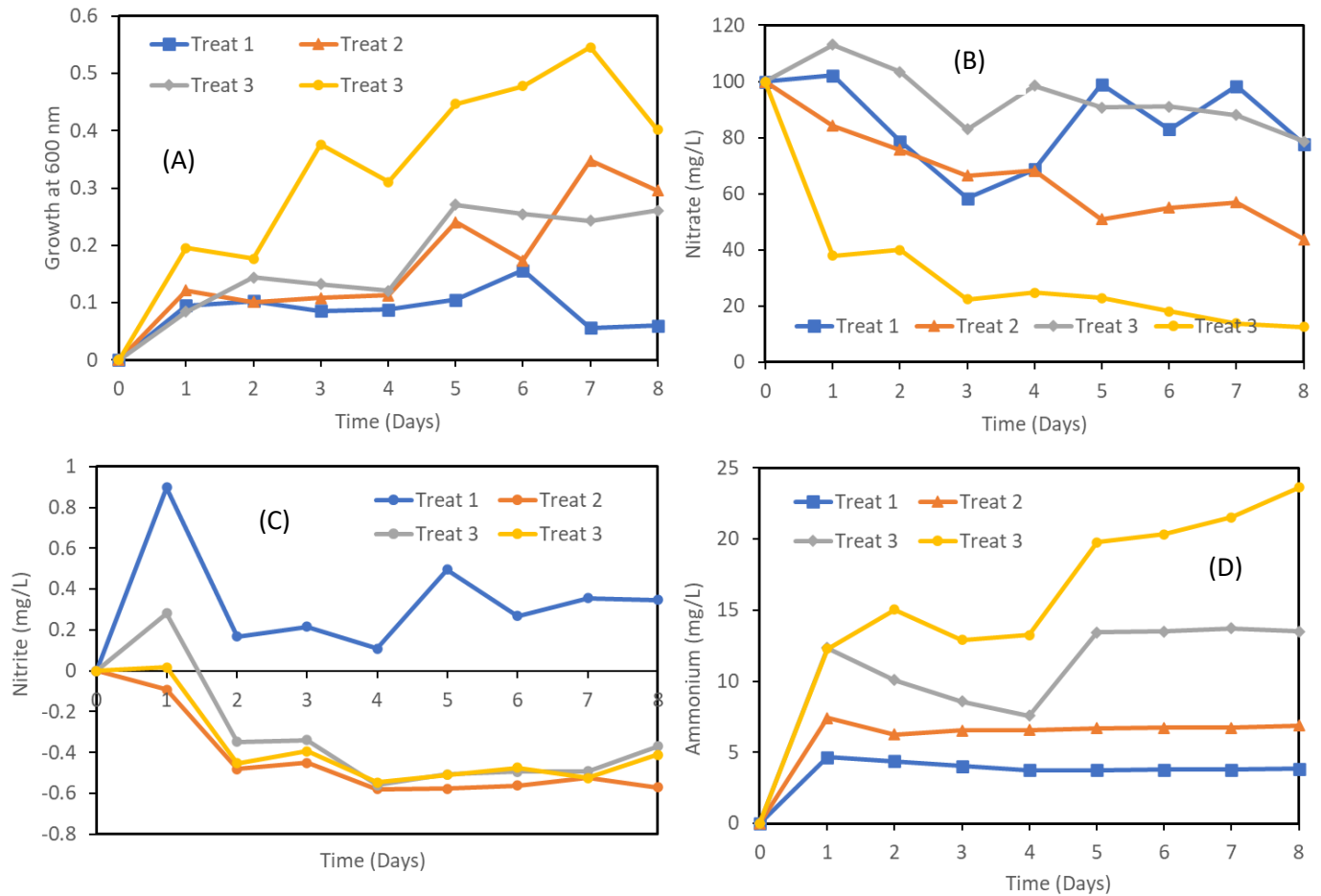


Fig. 4.7. Removal of nitrate in synthetic medium through a soil column approach (A) Bacterial Growth (B) Nitrate reduction (C) Nitrite Formation and (D) Ammonium Formation



5. DISCUSSION

5.1. Nitrate reducing heterotrophic bacteria

The different heterotrophic bacteria obtained from bioremediation laboratory of Periyar University were tested for its nitrate reducing ability using potassium nitrate broth. Among 10 bacterial isolates, *Priestia flexa* (GS1) was found to be efficient in nitrate reduction. Doudoroff and Palleroni (1974) selected a denitrifying bacterium, *Priestia* sp. like organism for nitrate removal because of its commercial availability, denitrification abilities, and physical versatility. It is commonly found in terrestrial soil and can grow in a variety of low nutrient conditions. Shanthi (2005) have isolated different heterotrophic denitrifying bacteria such as *Alcaligenes*, *Micrococcus*, *Bacillus*, *Corynebacterium*, *Pseudomonas* from lake water and sediment. Patureau *et al.* (2000) isolated many aerobic denitrifiers from various environments such as ponds, canals and soils or activated sludges and they belong to different genera *Paracoccus*, *Thiobacillus*, *Enterobacter*, *Comamonas* and *Sphingomonas*. Bacteria capable of denitrification are easily isolated from aquatic, sediment, and soil environments. *Acinetobacter* sp., *Aeromonas* sp., *Pseudomonas* sp., *Shewanella putrefaciens*, *Alcaligenes* sp. and *Achromobacter* were isolated from a hydrogenotrophic reactor and estuarine environments for the denitrification of drinking water (Selenka and Dressler, 1990; Liessens *et al.*, 1992). MacFarlane and Herbert, (1982) reported *Aeromonas* is one of the most prevalent nitrate respiring organisms in estuarine environments.

5.2. Nitrate removal

Physical, chemical and biological processes have been developed for nitrate removal from water. Although reverse osmosis, electrolysis and ion exchange are very effective in removing nitrate from contaminated water, they also result in concentrated waste brine, which must still be disposed of (Kesseru *et al.*, 2002). The other methods like ion exchange membrane bioreactor system, catalytic reduction, distillation, chemical denitrification and constructed wetland remediation currently have limited potential for full scale operation and / or their complex process on plant scale and the process is considered too expensive (Kapoor and Viraraghavan, 1997).

Sison *et al.* (1995; 1996) have studied a denitrification process by using granular activated carbon (GAC) column and dynamic mode of external carbon addition. This

process is suitable for water or wastewater containing NO_3 but insufficient organic matters. It was demonstrated that about 87 % of nitrate was removed by adding the organic matters once a day with continuous inflow of 20 mg.l^{-1} of nitrate. By using activated carbon also they can reduce the nitrate from synthetic solution and ground water but it creates disposal problem of solid waste. Due to these limitations for removal of nitrate from industrial waste water and ground water, the most versatile and widely used technology is biological denitrification (Mateju *et al.*, 1992; Kesseru *et al.*, 2002). Ground water with high nitrate content poses a serious threat to the environment and living beings. Microorganisms play a major role in the reduction of inorganic compounds. Hence biological treatment methods can be followed, which is ecofriendly and uses microorganisms and / or plants to convert the inorganic to some of its by products. Here aerobic microbial treatment combined with chemical treatment was attempted to remove nitrate from synthetic nitrate solution and ground water.

5.3. Nitrate reduction by aerobic heterotrophic bacteria

Nitrate, the most abundant form of nitrogen in the biosphere after atmospheric dinitrogen, is the N source used most widely by living organisms, including higher plants, algae, fungi and bacteria (Guerrero *et al.*, 1981; Stewart, 1988). Assimilatory nitrate utilization involves the reduction of NO_3^- to NO_2^- in a two-electron reaction mediated by nitrate reductase, and further reduction of NO_2^- to NH_3 involves six electrons in a reaction mediated by nitrite reductase (Guerrero *et al.*, 1981). Some facultative anaerobes are also able to use NO_3^- as an alternative electron acceptor in respiratory chains (Zumft, 1992). This inorganic form of nitrogen is a pollutant and its elimination is of high priority to environmental protection agencies. Its presence at relatively low concentrations usually does not limit its uptake, as most organisms have active NO_3^- uptake systems (Fossing *et al.*, 1995). Indeed complex microbial communities in biological treatment plants are able to cope with low concentrations of nitrate ($< 50 \text{ mg.l}^{-1}$) in a similar manner to those found in natural environments (Fossing *et al.*, 1995). Most denitrifying bacteria, however, are heterotrophic. They require an organic carbon source for cell growth and nitrate reduction. In the present study, the bacterial strains *Priestia flexa* (GS1), *Bacillus* sp. (GS2), *Alcaligenes* sp. (GS3), *Corynebacterium* sp. (GS4), *Bacillus* sp. (GS5), *Bacillus* sp. (GS6), *Bacillus* sp. (GS7),

Corynebacterium sp. (GS8), *Alcaligenes* sp. (GS9) and *Bacillus* sp. (GS10) were screened for nitrate reduction and *Priestia flexa* (GS1) was selected as best nitrate reducers. Nitrate reduction efficiency of GS1 was tested in synthetic medium with varying concentrations of different carbon sources.

5.4. Effect of carbon sources on nitrate removal

The studies on nitrate reduction from synthetic medium supplemented with different concentrations (0.5%, 1.0% and 1.5%) of carbon sources namely glucose, starch and cellulose with 100 mg/l of nitrate by GS1 was carried out under aerobic conditions. From the results of the above study conducted with different and various concentration of carbon sources (glucose, starch and cellulose) inoculated with GS1 the following criteria were drawn. The selected species GS1 was capable of removing maximum rate of nitrate in all the concentrations of carbon sources in synthetic medium. However, cellulose at 1% showed significant removal of nitrate amongst. The removal of nitrate was decreased when the concentration of cellulose at 0.5 and 1.5% concentrations. From the above results, it was inferred that, due to higher organic load the growth was affected. Hence, cellulose at 1 % concentration was used as carbon source for nitrate removal in the entire studies.

In the present study, a rapid of removal of nitrate was achieved in MSM supplemented with 1 % cellulose by GS1 at an optimum temperature of 35°C. To quantify nitrate reduction kinetics of bacterial isolate three different carbon sources were tested separately along with control without carbon source. The results showed that the carbon sources influenced the growth and higher level of nitrate reduction under aerobic condition. Methanol, ethanol and acetic acid were commonly used as organic substrates to provide the reducing power for nitrate elimination (Bockle *et al.*, 1986; Dahab and Young, 1988; Liessens *et al.*, 1993; Bohler *et al.*, 1994). Mateju *et al.* (1992) indicated that 3.0 kg of methanol was required to remove 1 kg of nitrate nitrogen rather than the stoichiometric amount of 2.47 kg. The results of this study demonstrated that all the bacterial isolates could utilize all the four carbon sources to achieve a high level of nitrate reduction (Kesseru *et al.*, 2002). Shanthi (2005) achieved nitrate removal in synthetic wastewater with 50 mg.l⁻¹ of nitrate amended with various carbon sources such as glucose, sucrose, cellulose and acetic acid by microbial consortium under

aerobic and anaerobic conditions. The results revealed acetic acid as the suitable carbon sources by bacterial consortium that reduced about 99.26 % of nitrate in synthetic wastewater.

It was observed that the carbon sources influenced the pH of medium during nitrate reduction under aerobic condition. There was very negligible pH change in synthetic medium with carbon sources during nitrate reduction under aerobic condition. The pH change was shown to be a clear indication of progress of nitrate reduction reaction, including the ability to discriminate between the negligible pH effects of nitrate reduction to nitrite and the pH increase was associated with the reduction of nitrite to non-ionic nitrogen products (Glass and Silverstein, 1998).

During nitrate reduction, accumulation of significant amount of nitrite and ammonium was observed in media, both with and without carbon sources. The accumulation of nitrite in bacterial culture may be in principle due to either assimilatory or dissimilatory nitrate reduction or due to heterotrophic nitrification. However, it is unlikely that nitrite accumulation results from nitrate assimilation since nitrate and nitrite reduction should highly coupled during assimilation. Under anoxic conditions, many species of bacteria are able to couple the generation of a transmembrane proton electrochemical gradient to the reduction of nitrate to nitrite, catalyzed by a respiratory nitrate reductase. The nitrite so generated can be further reduced either to ammonia, or to nitric oxide, nitrous oxide, and dinitrogen (Cole, 1988; Kuenen and Robertson, 1987). The nitrite reduction rate under aerobic conditions was very low and results from previous work with *A. faecalis* (Otte *et al.*, 1999) indicated that the observed N_2O production may not be due to reduction of NO_2^- , but may be a by product of heterotrophic nitrification. Low NO_3^- supply and a non-limiting supply of fermentable substrate favours rapid reduction to NH_4^+ , high NO_3^- and limited energy source appear to permit reduction only to NO_2^- . Recently, Kornaros *et al.* (1996) have shown that nitrite was accumulated in the cultures of *Pseudomonas denitrificans*, ATCC 13867 when nitrate was present at higher concentrations.

Many investigators (Tiedje, 1981; Wilderer *et al.*, 1987; Veydovec *et al.*, 1994; Kornaros *et al.*, 1996; Rijn *et al.*, 1996) have observed that nitrite can inhibit denitrification, especially at high concentration. Accumulation of extracellular nitrite has

been reported during denitrification in pure cultures implying that, under some conditions, denitrifying bacteria transport the nitrite intermediate out of the cell and later take the extracellular nitrite back in to the cell for complete denitrification. In aerobic condition the predominant fate of NO_3^- reduction is generally believed to be reduction to N_2O and N_2 by bacterial respiration (denitrification). In our experiments nitrate reduction has lead to the formation of nitrite and ammonium which means the bacterial genera of our study may have catalyze an alternative reduction pathway, dissimilatory reduction of nitrate to NH_4^+ (Payne, 1993; Scott and DeMoss, 1976). There is no fundamental argument why denitrification cannot occur under oxic condition. However, only during the past few years this activity received some attention (Berks *et al.*, 1995; Robertson *et al.*, 1995; Gupta, 1997; Patureau *et al.*, 1998).

The temperature effect on the denitrification rate is another important feature in the design of a denitrification process (Orhon *et al.*, 2000; Carrera, 2003). Konishi (1969) have reported incubation near 30°C generally, but not always favor denitrification. Thermophilic and psychrophilic denitrifying bacteria are known to have different temperature optima than do the mesophiles. High temperature optima reported were possibly a reflection of nitrobiological reactions (Bremmer and Shaw, 1958). Carrera (2003) have studied nitrate removal by activated sludge process in aerobic bioreactor at various temperatures (6, 8, 10, 15, 20 and 25°C). In this study he observed maximum nitrate removal at 25°C (79 to 86 %) after 24 hrs. In the present study, a maximum of nitrate reduction was recorded at 35°C whereas the percent removal was less when the study was carried out at lower temperatures. Because the lower and higher temperature might have affected the bacterial growth in synthetic medium containing nitrates.

6. SUMMARY AND CONCLUSION

Nitrate is wide spread in nature and is considered as a ubiquitous pollutant in ground water. High nitrate concentrations in drinking water have been blamed for several health problems which includes methemoglobinemia, goiter, birth defects, hypertension, anemia, stomach cancer, oral cancer, cancer of colon, gastrointestinal cancer, lymphoma and dyspnea etc. Elimination of nitrogen compounds such as nitrate nitrogen and ammoniacal nitrogen in ground water is considered crucial for remediation of the environment. To remove nitrate, heterotrophic bacteria are considered as ideal biological source. Hence, an attempt was made to remove nitrate from synthetic water using heterotrophic bacteria.

In the present study, the potential bacterial strains were obtained from bioremediation laboratory of Periyar University were identified to their generic level. The potassium nitrate broth medium was used in this study to find out its nitrate removal efficiency. The strains (GS1 to GS10) were grouped into +++ as high nitrate reducers, ++ as moderate nitrate reducers and + as low nitrate reducers. Potential strain GS1 was selected based on its synergistic activity against other bacterial strains (GS2 to GS10). The strain GS1 showed no antagonism with each other and hence it was selected for further nitrate removal studies. Molecular identification of the strains through 16S rRNA sequencing showed that the sequence results of GS1 with greater base pairs was similar to *Priestia flexa* (PP275113).

The effect of the carbon sources like glucose, starch and cellulose at various concentrations (0.5%, 1.0% and 1.5%) were tested on nitrate reduction using *Priestia flexa* (GS1). Simultaneously the bacterial growth, nitrite and ammonium formation were also estimated. Mineral salts medium incorporated with 100 mg/l of nitrate and varying carbon sources was used in this study in order to find out its nitrate removal efficiency. Among 10 bacterial strains GS1 was selected based on its nitrate reduction efficiency and physical nature of the strains. The growth of the GS1 in synthetic medium with different concentration of glucose (0.5%, 1.0% and 1.5%) containing nitrate was assessed. The growth of GS1 was maximum in 1% concentration of glucose in

synthetic medium supplemented with glucose (100 mg/l) in entire incubation period. The growth was maximum which as 0.627 OD after 6th day of incubation. The growth was slightly less in the MSM with glucose at 0.5 and 1.5% respectively.

The reduction of nitrate content in the synthetic medium containing various concentration of glucose (0.5%, 1.0% and 1.5%) by GS1 was tested. The GS1 reduced nitrate from 100 to 28.13 mg.l⁻¹ in synthetic medium with 0.5% glucose on 4th day of incubation. High nitrate reduction was noticed in 0.5% glucose supplemented synthetic medium compared to 1.0% and 1.5%. During nitrate reduction, formation of nitrite and ammonium in synthetic medium supplemented with various concentration of glucose was tested. The nitrite formation during nitrate reduction was high (2.9 mg/l) in synthetic medium amended with 1.5% glucose on 2nd day of the experiment. The amount of ammonium formed during nitrate reduction varied with glucose at various concentrations. The amount of ammonium formed was maximum (30.96 mg/l) in MSM supplemented with 1.5% glucose and 100 mg.l⁻¹ of nitrate. It was stagnant until the incubation period of 7th day.

The effect of the starch at various concentrations (0.5%, 1.0% and 1.5%) was tested on nitrate reduction. The GS1 reduced nitrate from 100 to 21.95 mg.l⁻¹ in synthetic medium with 1% starch on 4th day of the incubation. It was slightly increased after 4th day of the incubation period. The medium with 0.5% starch stands high nitrate reduction next to 1% concentration which was 100 to 41.73 mg/l. Starch at 1.5% concentration shown there was negligible reduction of nitrate. The nitrite formation during nitrate reduction was very high (3.71 mg/l) in synthetic medium amended with 1.5% starch on 2nd day of the experiment. It was suddenly decreased to 0.24 mg/l after 3rd day of the study. The nitrile level in the medium with 0.5 and 1.0% concentration of starch showed very insignificant. After 3rd day there was slight formation of nitrite was recorded in the medium with each concentration of starch. The amount of ammonium formed during nitrate reduction varied with starch at various concentrations. The amount of ammonium formed was maximum (30.81 mg/l) in MSM supplemented with 1% starch and 100 mg/l of nitrate. Thereafter the ammonium content was got decreased. It was

insignificant until the incubation period of 7th day. The amount of ammonium was shown maximum (30.96 mg/l) in the medium with 1.5% starch on the 3rd day and thereafter constant until 7 days incubation.

The effect of the cellulose at various concentrations (0.5%, 1.0% and 1.5%) was tested on nitrate reduction. The GS1 reduced nitrate from 100 to 9 mg.l⁻¹ in synthetic medium with 1% cellulose on 2nd day of incubation. Afterwords, there was a slight reduction of nitrate recorded. High nitrate reduction was also noticed in 0.5% and 1% concentrations of cellulose in the medium after 2nd day of incubation period. The nitrite formation during nitrate reduction was high (4.5 mg/l) in synthetic medium amended with 1% concentration of cellulose on 2nd day of the experiment. It was gradually decreased and got 0 mg/l after 6th day of the study. The nitrile level in the medium with 0.5 was very less. The formation of nitrite at 1.0% concentration of cellulose showed very negligible. After 2nd day, there was slight formation of nitrite was recorded in the medium with 1.5% concentration of cellulose. The amount of ammonium formed during nitrate reduction was varied with cellulose at various concentrations. The amount of ammonium formed was maximum (11.90 mg/l) in MSM supplemented with 1% cellulose and 100 mg.l⁻¹ of nitrate. It was decreased after 1st day of incubation.

Synthetic MSM was used with 100 mg/l of NO₃⁻ and supplemented with 1% concentration of varying natural substrate namely, coir wastes, orange fruit residues, corn fruit silks and sugar cane bagasse and sterilized. Nitrate removal was studied in this medium with 1% of GS1 inoculum and the results revealed that nitrate removal efficiency showed very negligible when natural substances used.

The effect of various temperatures (25, 30, 35, 40 and 45°C) on the removal of nitrate by the strain GS1 was carried out in MSM supplemented with optimized cellulose concentration of 1%. Among the study with different temperatures, 35°C exhibits maximum removal of nitrate. At the 35°C, the nitrate reduction was recorded from 100 to 31.88 mg/l on 3rd day of incubation period. Thereafter, the nitrate level was constant and showed only a little bit difference.

Since, the pH of the water samples is varied from place to place, the effect of various pH's (5, 6, 7, 8 and 9) on the removal of nitrate by GS1 was carried out in MSM supplemented with optimized cellulose concentration of 1%. Among the study carried out with different pH's, pH7 exhibits maximum removal of nitrate. This might be due to supports of hydrogen ion concentration for the removal of nitrate and also the growth of the bacterium.

With suitable optimized conditions, bioreactor study was carried out for the removal of nitrate (100 mg/l) from synthetic MSM. In order to find out the efficiency of nitrate removal by GS1, different concentrations (0.5, 0.75, 1.0 and 1.25%) of cellulose in the synthetic medium was used. The maximum removal of nitrate was recorded in the bioreactor containing MSM supplemented with 1% concentrations of cellulose. The nitrate reduction was higher (from 100 to 8.19 mg/l) on 6th day of treatment. After 6th day there was a slight increasing of nitrate recorded. There is no much difference on nitrate removal was recorded in other concentrations of cellulose. The nitrate removal was very negligible in control bioreactor. Hence, it could be accomplished that, the bioreactor approach can be a suitable eco-friendly technique for the removal of nitrate. The bacterially treated samples from each bioreactor were processed to remove microbial biomass and other solid materials through conventional coagulation process. After coagulation treatment with lime, the amount of nitrate, nitrite, ammonia and bacterial count were very trace level and attained its treatment significantly.

The bacterial strain GS1 was used in this study in order to find out its nitrate removal efficiency through a fixed bed soil column treatment 1 to 4. Removal of nitrate at a concentration of 100 mg/l was carried out through a column set up containing soil enriched with strain GS1. Among 4 different treatments studied, treatment 4 (soil with 1.5% cellulose and 1% inoculum) showed significant removal of nitrate from 100 to 37.91 mg/l on 1st day. Thereafter there was a gradual removal of nitrate. The study carried out with treatment 2 to 3 shown somewhat negligible removal of nitrate. This might be due to varying circumstances namely, environmental factors like temperatures, pH and room humidity. Formation of nitrite shown significant in the column treatment 1.

There is no nitrite formation recorded in treatment columns from 2 to 4. In each column treatment the formation of ammonium showed a gradual increase. However, the treatment column 4 containing soil and 1.5% concentration of cellulose shown maximum production of ammonium.

Depletion of ground water sources, contamination of ground and surface water by enormous usage of nitrogenous fertilizers, organic manures, human and animal wastes and industrial effluents and demand for drinking water sources, have made the researchers to find alternate methods to treat the ground water. Since, nitrate is one of the major pollutants in both surface and ground water, bacterial treatment with suitable bacterium like *Priestia flexa* (PP275113) was found to be effective for nitrate removal.

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